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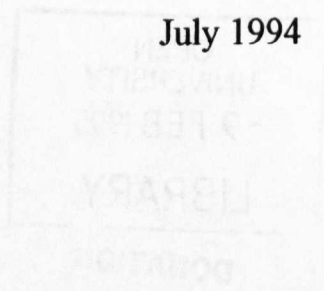
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VIRULENCE AND PATHOGENIC MECHANISMS OF
LEGIONELLA PNEUMOPHILA

by Ann Rawkins nee Williams

A thesis submitted in partial fulfilment of the requirements of the
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ABSTRACT

Strains of *Legionella pneumophila* which were virulent for guinea pigs were passaged on laboratory media such that they became avirulent. Virulent/avirulent pairs of strains were compared in an attempt to identify potential virulence or pathogenic factors.

Virulent forms multiplied within guinea pig alveolar macrophages maintained in tissue culture over 48 hours and phagosome lysosome fusion was not inhibited in these macrophages. Avirulent strains did not multiply but those produced by passage on supplemented Mueller Hinton agar (MHIH) maintained viability whereas those produced by passage on buffered charcoal yeast extract agar (BCYE) were killed. Virulent legionellae produced more flagella than their paired avirulent forms but it was considered unlikely that this was related to pulmonary virulence. Minor differences in outer membrane proteins and reactivity with various antisera were observed between virulent/avirulent pairs but these were not consistent between the *L.pneumophila* strains and no virulence-associated proteins were found. The lipopolysaccharide and extracellular enzyme activities of the pairs of strains were indistinguishable.

The Corby strain of *L.pneumophila* (CV) was isolated from its intracellular *in vivo* environment and these bacteria compared with the same strain grown on BCYE. The *in vivo*-grown CV showed no change in uptake by, or intracellular replication within, alveolar macrophages but, when Western blotted and incubated with various antisera, the two types of bacteria reacted differently. There was a reduced reaction by the CV grown *in vivo*, suggesting a change in the number and/or type of proteins expressed when exposed to an intracellular environment. The cellular responses in the lungs of guinea pigs immediately following aerosol challenge with virulent and avirulent forms of the Corby strain were compared. The avirulent forms evoked little change in the alveolar cell populations whereas an inflammatory response to CV occurred. Polymorphonuclear leucocytes (PMNLs) were the principal cell type involved and the initial increase in numbers of this cell type corresponded with a transient decrease in the viability of CV, providing evidence that virulent *L.pneumophila* are killed by PMNLs. The multiplication of CV which followed suggested a developing resistance to the killing. Numbers of avirulent Corby decreased rapidly and, for the MHIH passaged form (CA), this was in contrast to its intracellular survival *in vitro*. An attempt was made to follow the changes in cellular responses using immunolabelling techniques and flow cytometry. Difficulties with obtaining consistent, sequential samples did not allow a full interpretation of the results but the technique showed promise for such studies.

The tissue destructive protease (TDP) of *L.pneumophila* was shown to degrade or inactivate gamma interferon, IgG and possibly interleukin 2, proteins of possible significance to the host in protection against infection with *L.pneumophila*. Inhibition of TDP by the protease inhibitor α 2 macroglobulin was demonstrated and, prophylactic treatment of guinea pigs with the inhibitor resulted in prolonged survival (compared with untreated controls) following aerosol challenge with CV. Intracellular production of TDP by *L.pneumophila* multiplying within guinea pig alveolar macrophages was demonstrated by ELISA and immunogold labelling and functional activity of the enzyme purified from infected guinea pig lungs was shown. A mutant of *L.pneumophila*, deficient in TDP production, was compared in the guinea pig model with its TDP-producing parent and CV. The mutant and parent were considerably less virulent than CV but, despite the deficiency in TDP production, the mutant was lethal for guinea pigs. The lung damage caused by the mutant was less severe than that caused by the parent or CV and it was suggested that progression of disease and cause of death was not typical of Legionnaires' disease. An immunocompromised mouse model of Legionnaires' disease was investigated using aerosol infection of severe combined immune deficient (SCID) mice. SCID mice did not become ill when given a potentially lethal aerosol of *L.pneumophila* and alveolar macrophages of these mice did not support the growth of *L.pneumophila in vitro*. SCID mice which had been reconstituted with human leukocytes were susceptible to aerosol challenge and developed lung lesions similar to those seen in guinea pigs and humans.

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DEDICATION

To Davey

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CHAPTER 1

General Introduction to Legionnaires' disease and *Legionella pneumophila*

1.1 HISTORICAL BACKGROUND

Legionnaires' disease (LD) is an acute bronchopneumonia caused by certain members of the *Legionella* genus but most commonly by *L.pneumophila*. First identification of the causative agent occurred in the fairly recent past in 1976 following an outbreak of bronchopneumonia at the Legionnaires' conference in Philadelphia (Fraser *et al*, 1977). Since then retrospective serology showed that LD was not a new disease and that many past deaths due to pneumonia of unknown aetiology were in fact a result of LD (Thacker *et al*, 1978, CDC, 1977). Once a suitable culture medium had been found (McDade *et al*, 1977) more cases of LD were discovered and it soon became apparent that *L.pneumophila* was a cause of both community acquired and nosocomial pneumonia (Broome *et al*, 1979). A pattern of patient susceptibility emerged with the main risk groups being the elderly and immunocompromised individuals.

Not long after the discovery that *L.pneumophila* causes LD the same organism was shown to be responsible for an outbreak of a flu-like illness that had a similar aetiology to LD with its dissemination from environment to patient but the resulting disease was quite different. The condition termed Pontiac Fever after the site of the first recorded outbreak (CDC, 1968, Glick *et al*, 1978), is a milder, non-invasive, non-pneumonic, febrile disease with many poorly defined symptoms that make epidemiological studies difficult (Glick *et al*, 1978). The disease is characterised by a much higher attack rate than LD and is not restricted to certain population groups. It is not known why the same organism

causes two distinct diseases. It is unlikely to be simply a milder form of the same disease because cases of Pontiac fever are not associated with LD outbreaks and vice versa (O'Mahony *et al*, 1990, Goldberg *et al.*, 1989).

The Philadelphia 1 strain of *L.pneumophila* which caused the original outbreak was designated serogroup 1 as strains, which were later isolated from water systems and patients, did not cross react with sera raised against this first isolate. To date 14 serogroups of *L.pneumophila* have been identified but serogroup 1 is by far the most common cause of Legionellosis with 50% of cases due to serogroup 1 and 10% to serogroup 6 (Reingold *et al*, 1984). Other *Legionella* species are known to cause LD and Pontiac Fever. Several of these are regular disease producers e.g. *L.micdadei*, *L.bozemanii*, but many have been identified as a result of being responsible for isolated outbreaks or cases e.g. *L.longbeachae* (Steele *et al*, 1990), *L.sainthelensis* (Garcia *et al*, 1992). Recent data showed that of the 32 *Legionella* species only half have been reported as pathogenic for humans, the rest being isolated from environmental sources (Benson *et al*, 1991). However, a survey of potable water systems in the U.K. (Dennis *et al*, 1982) revealed that legionellae that were not associated with human disease were nonetheless virulent for guinea pigs indicating that a potential source of legionellosis exists wherever legionellae are found.

The bacterium's natural habitats are water and soil and virtually any means by which aerosolisation of such habitats can happen have lead to the occurrence of LD. The most common sources of infectious aerosols are man-made water systems particularly cooling towers, hot water systems, humidifiers and whirlpool baths (Breiman, 1993).

Extensive surveys carried out in several countries revealed that the bacteria could successfully colonise man-made water systems even if these were well maintained (many were not). It was clear that

legionellosis had to be considered in the construction and maintenance of such systems. Guidelines and Codes of Practice have now been produced (HSE, 1991) which should decrease the incidence of LD considerably. However, as one source of infection is being tackled others are coming to light. The recent popularity of whirlpool baths for example has lead to many cases of LD and Pontiac Fever.

The incidence of Legionellosis in England and Wales appears to be falling, possibly as a result of more public awareness and the damaging consequences to employers and government of preventable outbreaks. The problem is larger in the USA where an estimated incidence of pneumonia due to legionellae is 1/20,000 people annually (Marston *et al*, 1993). Fortunately, large outbreaks are becoming less frequent but community acquired pneumonia continues to be a problem. Sporadic cases of LD are difficult to evaluate epidemiologically and, although many are travel-associated, the danger that a sporadic case may be the start of an outbreak means that despite falling numbers of cases it is vital to maintain public awareness and vigilance for a potentially dangerous disease.

1.2. CLINICAL FINDINGS, DIAGNOSIS AND TREATMENT.

Legionnaires' disease is often described as an atypical pneumonia but this is possibly a misnomer since there are few clinical features that clearly distinguish LD from other pneumonias or indeed conditions other than pneumonia. The early symptoms of infection are similar to those of influenza, i.e. headache, fever, chills and muscle pain. The illness that follows often exhibits extra-pulmonary symptoms involving the gastrointestinal tract, central nervous system and kidneys. During an outbreak such as that which occurred at the BBC where individuals

dispersed from the source of infection, the non-specific nature of symptoms is a problem, particularly in the early stages of infection.

The yardstick of diagnosis of LD is demonstration of the bacterium in lung tissue either by culture or by immunostaining. Culture is the most sensitive method, in that one viable bacterial cell can be detected but this level of sensitivity is dependent on the culture medium and difficulties with quality control of media, particularly between laboratories, creates a problem. These difficulties are being recognised and better standardised media are being sought. A major problem with culture methods is the danger that the portion of respiratory tract being cultured does not contain any organisms since the infection can be very localised. Increasingly there are cases of LD caused by strains that are not culturable on laboratory media (Hussong *et al*, 1987) but which will multiply in protozoa (Rowbotham, 1993). These so called *Legionella*-like amoebal pathogens (LLAPs) have now been confirmed as belonging to the *Legionella* genus by 16S ribosomal RNA typing (Rowbotham, 1993). Thus, perhaps culture methods should include incubation of specimens with amoebae. Demonstration of *Legionella* antigens in lung tissue (obtained by biopsy) by immunochemical techniques is a very specific test and does not require viability of the bacteria. It has proved useful in environmental sampling but has a low sensitivity in that several bacteria need to be present for a confirmatory result.

The most recent development in diagnosis of LD is detection of *Legionella* DNA by polymerase chain reaction (PCR). This method requires only one bacterium to be present in the sample and is potentially as sensitive or more sensitive than culture methods but given the above mentioned difficulties with culture, PCR would seem more promising. As yet sensitivities equivalent with culture are not being

achieved (Tompkins, 1993) and specificity is also a problem but as applications of this technique are expanding it is likely to improve and become more widely used to detect legionellae.

Direct demonstration of identifiable legionellae in respiratory specimens is the ideal diagnosis when possible but often the disease can only be diagnosed by demonstration of specific antibody in patients' sera. An indirect immunofluorescence antibody test (IFAT) is used and a four-fold rise in antibody titre (over 3-6 weeks) or a single high titre is required as positive diagnosis. Production of high levels of antibody can take time especially in an immunocompromised patient. This delay could be a serious problem for a patient requiring urgent specific treatment. Another problem with serology is that of cross reactions. Patients can develop antibodies that cross react with antigens of other pathogens (e.g. *Rickettsia*, *Mycobacteria*, *Bacteroides* and *Pseudomonas* species) or species and serogroups of *Legionella* other than that causing disease. The problem appears to be worse when disease is due to strains other than *L.pneumophila* serogroup 1. This may be a reflection of the antigens used to perform the serology since they are better characterised for serogroup 1. The main problem with serology is that methods are not standardised and different laboratories use a variety of antigen preparations and control sera. Improvements in methodology and adoption of standard procedures will enhance the usefulness of serology but the delay factor of antibody development will always be a drawback to the diagnosis of LD by this technique.

A diagnostic technique that has been studied for many years is detection of specific antigen in non-pulmonary body fluids the most convenient of these being urine (Berdal *et al*, 1979, Tilton, 1979, Mangiafico *et al*, 1981). Antigen appears in urine early on in the disease long before antibody production (Williams & Featherstone, 1988)

and in quantities sufficient to be reliably detected by sensitive, quantitative techniques such as radioimmunoassay (RIA, Kohler, 1981) and enzyme linked immunosorbent assay (ELISA, Berdal *et al*, 1979, Williams & Featherstone, 1988). Several kits for urinary antigen detection are available but have not yet become part of the standard procedures in hospitals. This is partly due to poor performance of latex agglutination assays having low sensitivity and specificity (Leland and Kohler, 1990) and reluctance to use RIA methods. However ELISA kits have given very promising results in several laboratories and should provide a diagnostic test of the future particularly where the assay has been improved to detect antigens from serogroups and species other than *L.pneumophila* serogroup 1 (Tang & Krishnan, 1993, Williams and Lever, unpublished).

Early identification of a case of LD is vital to curtail outbreaks but it is more important to administer the most effective antibiotic therapy. Legionellae are intracellular pathogens so the antibiotic used must be bactericidal and be able to penetrate the alveolar macrophages in which the bacteria multiply (Winn *et al*, 1978). The first antibiotic used to treat the disease was Erythromycin (Kirby *et al*, 1980) and is still in use today although there is some doubt about its efficacy both in patients (Miller, 1981) and in experimental animals (Fitzgeorge, 1985, Fitzgeorge *et al*, 1986). Erythromycin is less able to prevent intracellular growth of *L.pneumophila* than other antibiotics such as Rifampicin and Ciprofloxacin which successfully arrest the progress of acute experimental airborne LD (Fitzgeorge *et al*, 1986).

Prevention of LD is a multi-component problem. Ideally, the source of infection should be eradicated, which means prevention of legionellae colonising any water system from which aerosols can be produced. Since such systems include domestic water supplies where taps and shower heads provide the aerosol it would seem an enormous

task to undertake. Clearly in public buildings, especially hospitals, there is no question that all steps must be taken to prevent colonisation but as more is discovered about this organism in the environment the more extensive the procedures become. With the increased incidence of sporadic LD where each case can be due to a different set of circumstances, control of disease rests more heavily on rapid diagnosis and effective treatment.

1.3. LEGIONELLA PNEUMOPHILA

Classification of the *Legionella* genus has become extremely complicated over the years and strains can now be classified by serology, ribotyping (Saunders *et al.*, 1988, Grimont *et al.*, 1989), plasmid type (Stout *et al.*, 1988), restriction fragment length polymorphism (RFLP, Saunders *et al.*, 1990) electrophoretic alloenzyme typing (Selander *et al.*, 1985, Tompkins *et al.*, 1987), quinone analysis (Karr *et al.*, 1982, Wait, 1988) together with other techniques. Serology is the most commonly recognised basis for categorisation of legionellae but the increasing number of monoclonal antibody panels which distinguish sub-groups of *L.pneumophila* serogroup 1 has led to a very complicated system of typing. The confusion created by the different antibody types led to development of alternative methods of classification. Techniques such as electrophoretic alloenzyme typing and quinone types have separated the strains which overlap by serology but are not methods easily adopted by many laboratories. A successful typing technique is RFLP which distinguishes strains by DNA fragment patterns following restriction enzyme digestion and is a means of clearly matching clinical with environmental strains (Saunders *et al.*, 1990). Serology remains the widely used technique which will undoubtedly

change as monoclonal antibody stocks run down and more laboratories adopt genetic methods for diagnosis and epidemiology.

1.4. ECOLOGY

L.pneumophila is a fastidious organism that utilises amino acids as its carbon source having a particular requirement for iron and cysteine. Legionellae have been found in a wide variety of natural water habitats (Lee and West, 1991) including lakes, rivers, estuarine and marine waters and in pockets of water on plants in rain forests. They are also found in moist soil. A preference for warmer water (between 20-40°C, Dennis *et al.*, 1984, Wadowsky *et al.*, 1985) is the reason there is a greater number found in man-made water systems. In a number of these water environments nutrients are scarce and the legionellae have to occupy a particular ecological niche to survive. In recent years much attention has focused on the ability of legionellae to survive and multiply in single celled protozoa such as amoebae. It was originally reported by Rowbotham (1980) that legionellae could be found in the trophozoites and within cysts providing protection from adverse environmental conditions. A number of amoebal species can be infected as well as species of *Tetrahymena*. Rapid multiplication of legionellae has been observed and so amoebae and related organisms provide not only protection but also may provide a reservoir for its maintenance in aquatic habitats

Another niche for legionellae in the environment is in biofilms which are crusts of micro-organisms which colonise surfaces in natural or man made water systems. The diversity of organisms in a biofilm means that by-products of one organism may be raw materials for another for example, *Cyanobacteria* and green algae have been shown to support the growth of *L.pneumophila* in basal culture media (Tison *et al.*, 1980, Hume & Hann, 1984). Bacteria such as *Flavobacterium* and

Pseudomonas spp. can similarly promote growth of *L.pneumophila* though this has not been proven to occur in the environment (Lee and West, 1991). However, Lee and West, (1991) found that when the micro flora from a water sample containing *L.pneumophila* was inoculated into sterilised tap water in a chemostat model, the legionellae persisted in mixed culture for over two years. Biofilms can also provide protection for legionellae from water currents and biocides.

1.5. VIRULENCE AND PATHOGENESIS

L.pneumophila is a recently discovered pathogen but due to the severity of the disease and the large outbreaks which have occurred, a great deal of research effort has been devoted to elucidating the virulence and pathogenesis of the organism. A brief overview is given here since many of the topics are covered in the Chapters that follow.

L.pneumophila is an intracellular pathogen and is now known to be able to replicate in many cell types at least *in vitro* (Cianciotto *et al*, 1989b). If phagocytosis of *L.pneumophila* is prevented no replication takes place *in vitro* (Elliott and Winn, 1986). Outer membrane proteins of the organism have been widely studied and candidates for the mediators of attachment uptake and intracellular protection have been proposed. Many proteins have yet to be allocated a role. Excreted proteins, in particular enzymes, have been discovered which are toxic to the host and can modify host defence mechanisms but the absolute dependence on the production of these for virulence has not been shown.

In lungs, *L.pneumophila* multiplies within alveolar macrophages and much of the recent research is based on the possible mechanisms by which the bacterium resists the anti-microbial defences of this cell. Evidence of resistance to the respiratory burst of macrophages has been found but as yet it has only been demonstrated in *in vitro* experiments. The discovery

that *L.pneumophila* Philadelphia 1 strain inhibited the fusion of lysosomes with phagosomes containing the bacteria has been previously accepted as true for all virulent legionellae (Horwitz, 1983a, Dowling, *et al.*, 1992). As more and more strains are being studied differences between these and the Philadelphia 1 strain are emerging and not all strains appear to inhibit phagosome-lysosome fusion (Rechnitzer and Blom, 1989). This implies that such strains must be resistant to the lysosomal contents but due to the wide acceptance of phagosome lysosome fusion inhibition an alternative mechanism of resistance has not so far been reported.

Virulence factors without which the organism is unable to cause disease have yet to be clearly identified for *L.pneumophila* and in a recent review it was proposed that the reason is because there is no single factor (Engleberg, 1993). Avirulent forms of *L.pneumophila* have been extensively studied in order to identify such a factor and mutants lacking a particular factor have been reported to have reduced virulence. Examples of these modified bacteria are described in the following Chapters.

1.6. ENVIRONMENTAL FACTORS IN VIRULENCE

It is well established that the source of infectious legionellae is water but that the mere presence of the bacteria is not necessarily a precursor to disease. In order to cause disease the organism must not only have the inherent ability to invade and escape host defences but must exhibit equally important environmental virulence factors. These are principally the ability to survive and multiply in the environment (i.e. the source of infection) and to survive conditions on the route to the host. Most frequently the dissemination is by aerosolisation and parameters which affect the survival of legionellae within aerosol particles are critical in determining whether infection occurs. The size of the aerosol particles is also critical since these must be small enough (3.5µm in diameter) to reach

the lower respiratory tract. Larger particles containing virulent *L.pneumophila*, administered intra-nasally, do not produce Legionnaires' disease (Fitzgeorge *et al.*, 1983). The fact that a relatively small proportion of all the *Legionella* species and sub-species which have been isolated are associated with human disease is probably a reflection of the conditions in the environment rather than inherent virulence of the bacteria. A number of environmental factors have been shown to affect the potential to cause disease including temperature of the water (Dennis *et al.*, 1984 Mauchline *et al.*, 1992 & 1994), the ability of the organism to survive in aerosols which depends on the relative humidity of the air (Berendt, 1980 , Hambleton *et al.*, 1983,) and physical characteristics of the bacteria such as growth phase (Hambleton *et al.*, 1983) and surface hydrophobicity (Dennis, 1986, Dennis and Lee, 1988). An interesting feature of LD is that a particular strain or species will continue to cause outbreaks or sporadic cases in a particular geographical area, be that a building, a town, or a country but that strain rarely causes disease elsewhere. Such a strain is able to exploit a particular niche but its ability to be pathogenic is restricted to that set of circumstances. This epitomises the opportunistic nature of this genus which makes LD so difficult to predict and control.

1.7. RESISTANCE OF HOST TO LD

Cell-mediated immunity is reported to play an important role in defence against LD (Horwitz, 1983b, Friedman, 1988). Much of the evidence for this is based on *in vitro* studies or on the responses of guinea pigs to sub lethal infections. Avirulent *L.pneumophila* strains have been reported to induce cellular immunity in guinea pigs following aerosol challenge (Blander, 1989).

Much information is available about mechanisms by which the host may prevent uptake and replication of *L.pneumophila* mostly requiring activation or up-regulation of macrophage function. *In vitro* studies have shown a number of ways of stimulating this cell to inhibit intracellular growth including non-specific activation by LPS (Egawa *et al*, 1992) and by T-cell derived factors (Nash *et al*, 1984, Nikaido *et al*, 1989, Yamamoto *et al*, 1992) in particular gamma interferon (IFN γ) (Nash *et al* 1988, Jensen *et al* 1987). Vaccination of guinea pigs with *L.pneumophila* and its growth products has been shown to induce cell-mediated protection against aerosol challenge and non-specific stimulation of cellular immunity is also protective (Gibson *et al*, 1985, Yoshida *et al*, 1987). However there is no evidence that macrophage activation occurs in humans or experimental animals which have not been pre-treated or immunised.

The aim of the studies reported in this thesis was to contribute to the understanding of the virulence and pathogenesis of LD by investigation of the interaction of virulent and avirulent strains of *L.pneumophila* with a guinea pig host using a model of infection which delivered the organisms to the animals as a fine particle aerosol. Chapter 2 is an investigation of the strains *in vitro* with reference to possible virulence and pathogenic factors. Studies of the behaviour of the strains *in vivo* and the host response following airborne challenge are described in Chapter 3.

Previous studies by the author into the role of *L.pneumophila* protease in pathogenesis were extended in the light of reports that the enzyme was not required for virulence, the results are given in Chapter 4. The final chapter investigates a novel animal model of LD and describes the behaviour of a virulent strain in immunocompromised mice.

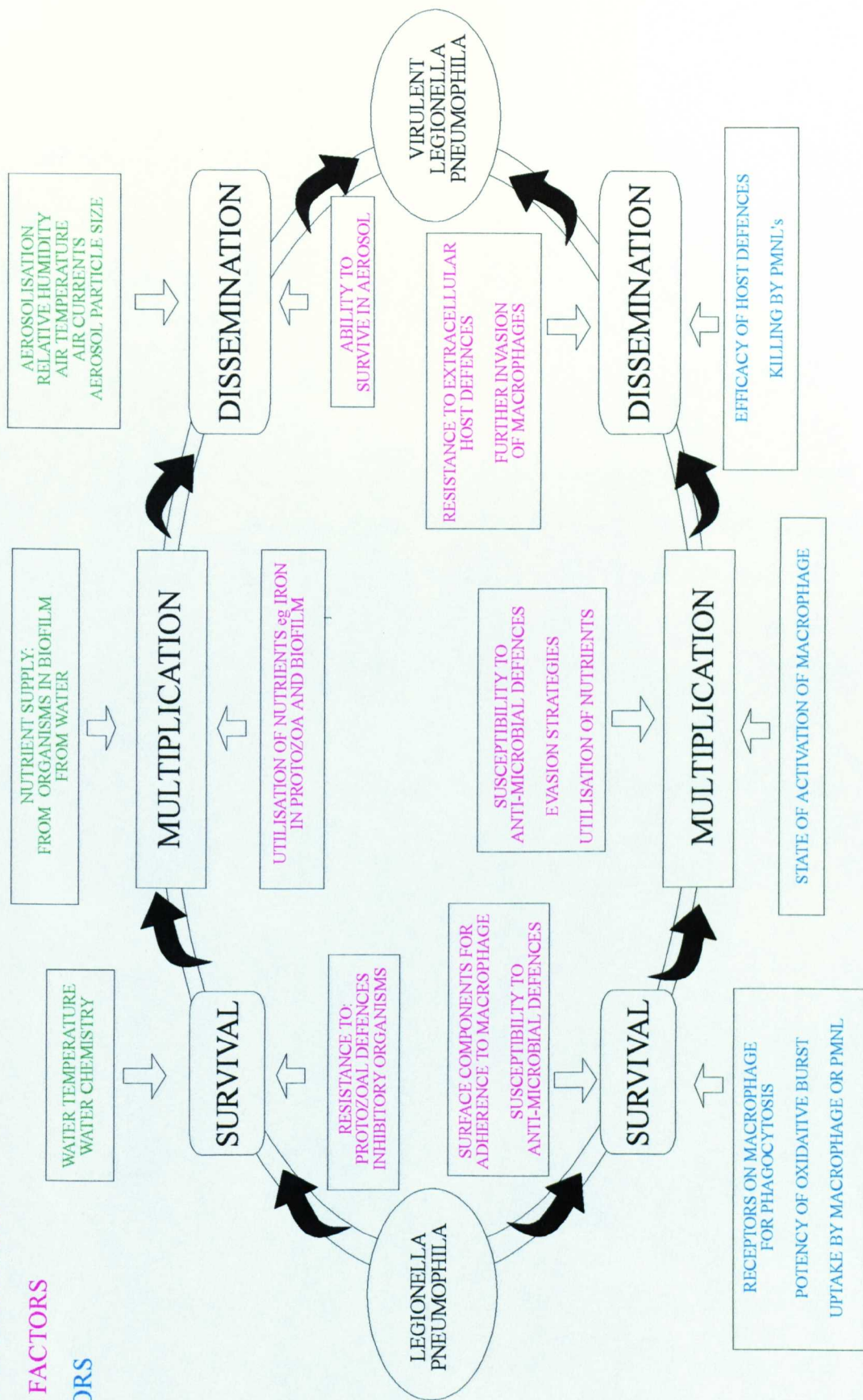
pathogenic factor or mechanism is one which facilitates or aids progression or severity of infection but is not necessarily an absolute requirement to cause disease. A virulence factor is one without which the bacterium would not cause disease.

Fig.1. Pathogenic / virulence factors involved in Legionnaires' disease

ENVIRONMENTAL FACTORS

BACTERIAL FACTORS

HOST FACTORS



CHAPTER 2

A comparison of virulent and avirulent forms of *L.pneumophila*

2.1. INTRODUCTION

When *Legionella pneumophila* was identified as the causative organism of Legionnaires' Disease (LD) one of the first culture media found to support its growth was Mueller Hinton agar supplemented with haemoglobin and isovitalect (MHIH) (Feeley *et al.* 1978). It was later discovered that strains recovered from MHIH were avirulent and several workers have reported this conversion as evaluated in various animal and *in vitro* models of LD virulence. McDade and Shepard (1979) reported that the Philadelphia 2 strain of *L.pneumophila* which had been passaged for 6 months on MHIH was less virulent in embryonated eggs than the same strain propagated through either guinea pigs or yolk sacs and the MHIH passaged strain was completely avirulent for guinea pigs when administered intra-peritoneally (i.p.). Organisms passaged through guinea pigs did not grow on MHIH agar. Wong *et al.* (1981) compared a clinical isolate of *L.pneumophila* which had lost its virulence for guinea pigs following culture on MHIH agar with a different strain which had been maintained in embryonated eggs. After 3 passages in cultured human lung fibroblasts the media-attenuated strain reverted to a virulence equivalent to the egg-grown strain. Elliot and Johnson (1982) also demonstrated avirulent to virulent conversion of MHIH grown bacteria following repeated passage through guinea pigs and, to a lesser extent, through embryonated eggs. Catrenich and Johnson (1988) have since reported that this reversion to virulence did not occur if the culture was passaged 25 times on MHIH or if a single colony growing on MHIH was tested. This suggests that the virulence switch is irreversible

and in the previous studies, passage of avirulent suspensions through guinea pigs or eggs resulted in selection and enrichment of a few remaining virulent cells within the cultures. A study by Horwitz (1987) showed that passage on MHIH of the Philadelphia 1 strain of *L.pneumophila* resulted in forms which were avirulent in guinea pigs and were also unable to multiply in cultured human monocytes due to an inability to inhibit phagosome lysosome fusion. When the outer membrane proteins, extracellular enzymes and serum resistance of the avirulent bacteria were compared with the virulent parent strains there were no differences which would account for the virulence switch. Repeated subculture on buffered charcoal yeast extract (BCYE) agar can also convert strains to avirulence (Jepras *et al.*, 1985). Despite the availability of such avirulent forms of *L.pneumophila*, the molecular mechanisms which account for the change in virulence are largely unknown.

Avirulent forms of pathogens provide useful tools for elucidating the pathogenic mechanisms of the virulent organism but it is vital, when comparing virulent with avirulent organisms, that the method of characterisation of virulence is relevant to the natural disease. Many of the studies of avirulent forms of *L.pneumophila* have involved models of disease which do not follow the usual route of infection. All used i.p. administration of the organism into guinea pigs or inoculation of hens eggs (Elliot & Johnson, 1982). An aerosol infection guinea pig model of LD developed by Baskerville *et al* (1981) has been well established as closely resembling the disease in humans. The use of such a model allows virulence of strains to be compared reliably using the natural route of acquisition/infection. The mechanisms by which the virulent strains evade host defences and why the avirulent strains are susceptible, can also be investigated. Since the alveolar macrophage is the primary component

of the host with which *L.pneumophila* interacts, guinea pig alveolar macrophages obtained by lung lavage provide a means of studying the interaction of these cells with various strains more closely *in vitro*.

Avirulent forms of bacterial pathogens often lack a vital virulence factor without which disease cannot be established. Such virulence factors include toxins, enzymes, lipopolysaccharide (LPS), outer membrane proteins, flagella and pili.

The aim of the following study was to examine attenuated forms of clinical isolates of *L.pneumophila* which had been passaged on MHH and BCYE agars and compare them with each other and with the original strain in terms of virulence for guinea pigs and infectivity for guinea pig alveolar macrophages and expression of factors likely to be involved in virulence and pathogenesis. The results of these comparisons will be discussed with reference to putative pathogenic mechanisms which have been suggested for *L.pneumophila*.

2.2. MATERIALS AND METHODS

2.2.1. ORGANISMS

Human isolate strains of *Legionella pneumophila* serogroup 1 were originally isolated on BCYE and stored at -70°C. The strains used were all clinical isolates from patients with Legionnaires' disease. The Corby strain was kindly provided by Dr.R.A.Swann (John Radcliffe hospital, Oxford, U.K.), Stafford was the strain which caused an outbreak at Stafford General Hospital and Dodge was isolated from a patient at the PHLS laboratory, Portsmouth.

Passage of these strains on laboratory media is depicted in Fig.2.1

2.2.2. MEDIA

Bacterial culture media used in the study were: buffered charcoal yeast extract agar (BCYE, Edelstein, 1981); Mueller Hinton supplemented with haemoglobin and "Vitox" (Oxoid, SR90) (MHIH, Feeley *et al.*, 1978); yeast extract broth (YEB, Ristroph, 1980). Tissue culture medium was modified Eagles medium (MEM, Imperial Laboratories) supplemented with 0.075 % sodium bicarbonate and 20mM HEPES buffer.

2.2.3. ANIMAL MODEL

The aerosol infection guinea pig model used in the characterisation of virulence of strains was that described by Baskerville *et al.* (1981). Female guinea pigs were exposed to an aerosol < 5µm in diameter, generated from water suspensions of *L.pneumophila* with a 3-jet Collison spray by means of a Henderson apparatus. Viable counts were performed on macerated lungs taken immediately after spraying

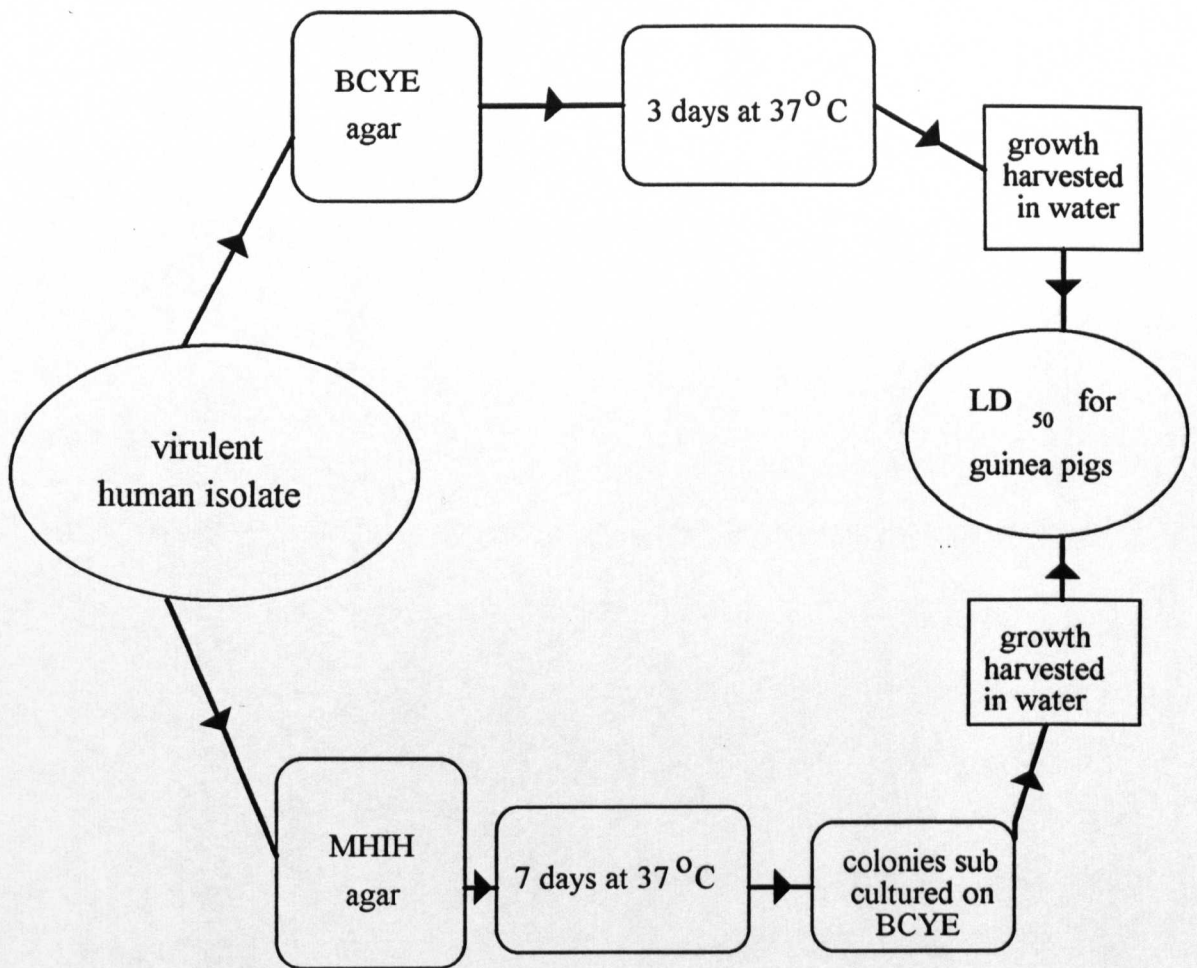


Fig.2.1. Passage of *L.pneumophila* strains on BCYE and MHIH agars in order to derive avirulent forms.

(0 hours) to determine the numbers of legionellae retained in the lungs. The animals were observed for signs of illness e.g. weight loss and increased temperature, and deaths were recorded. The viable counts at 0 hours and subsequent animal deaths were used to calculate LD₅₀'s by the Reed and Muench method (Reed and Muench, 1938).

Multiplication of the legionellae following aerosol challenge was monitored by viable counts on macerated lungs taken from individuals in a group of guinea pigs at intervals post infection.

2.2.4 GUINEA PIG ALVEOLAR MACROPHAGES

Lungs of normal, guinea pigs were washed out post-mortem in tissue culture medium (MEM) containing 5 U heparin ml⁻¹ and 20 mM L-glutamine using a procedure described by Jepras *et al.* (1985). Cells were harvested from the lavage by centrifugation at 200g for 10 min and resuspended in MEM containing 5% autologous guinea pig serum (MEMb). Cells were counted in a Neubauer haemocytometer chamber, adjusted to 1×10^5 - 10^6 macrophages /ml and 200µl aliquots added to wells of a 96 well tissue culture plate (Nunc) and allowed to settle at 37°C for 30 min. Non-adherent cells were then washed away with MEMb.

2.2.5. INFECTION OF GUINEA PIG ALVEOLAR MACROPHAGES

Bacterial suspensions were quantified by viable count on BCYE, suspended in MEMb and added to macrophages at a concentration which yielded 100 organisms/macrophage. A 2 hour period of incubation at 37°C was allowed for ingestion of organisms after which extracellular bacteria were killed by incubating for 2 hours with 50 µg/ml gentamicin in MEMb followed by 3 washes with MEMb, a procedure which does not kill intracellular *L.pneumophila* (Fitzgeorge, 1985). To

count intracellular bacteria the macrophages were disrupted by adding 200 µl 0.8% (w/v) digitonin in sterile distilled water to each well and incubating for 10 mins at 37°C. This detergent has been shown previously to have no effect on the viability of *L.pneumophila* (Fitzgeorge, 1985). The contents of each well were aspirated and added to 1.8 ml of distilled water. This suspension diluted further as necessary was plated onto BCYE agar. Extracellular organisms were enumerated by plating out serial dilutions of culture supernatants. Intracellular growth of organisms was investigated by incubating infected macrophages at 37°C for 24 and 48 hours followed by washing the macrophage culture with MEMb and then disruption by digitonin treatment and enumeration of bacteria by viable counts. Colonies were counted after 3 days incubation at 37°C in all cases. Controls were included to determine the effects of digitonin and MEMb on the growth of bacteria. During the course of infection macrophages were examined under phase contrast microscopy. To compare uptake of the strains by macrophages the bacterial suspensions were incubated with the cell sheet for 20, 40, 60, 80 100 and 120 min and intracellular counts performed as above.

2.2.6. GROWTH OF STRAINS IN GUINEA PIG LUNG LAVAGE

The lungs of normal and *L.pneumophila* infected guinea pigs were washed out with 0.12M potassium chloride (since sodium chloride has been shown to be inhibitory to *L.pneumophila*, Feeley *et al.*, 1979) and centrifuged to remove all cells. Lavages from infected animals were filtered through a 0.22µm diameter filter (Amicon) to remove all the organisms. A concentrate was produced by using a lavage fluid which had been taken from one guinea pig to successively wash out the lungs of 4 more guinea pigs. Lavage fluid was added to the wells of a 24 well tissue

culture plate (Nunc) and known concentrations of the various strains added. Viable counts of the wells were performed at 0, 24 and 48h.

2.2.7. PHAGOSOME LYSOSOME FUSION

Guinea pig macrophages washed out of normal guinea pig lungs were counted and characterised and adjusted to a concentration of approximately 1×10^6 cells /ml in MEMb. Aliquots of 0.5 ml were incubated with lysosome markers at 37°C in siliconised glass tubes (to prevent macrophages adhering) for various lengths of time to determine an optimum uptake period. The lysosome markers used were, thorium dioxide (Thoria sol, Polysciences) and colloidal gold (1 nm particle size) stabilised with bovine serum albumin (BioCell). Excess marker was washed off and the cells incubated for a further 3 hours to allow all the marker to be internalised. Bacterial suspensions were added as above and the cells incubated for 6 and 20 hours. Incubation was stopped by placing the cells in ice. The cells were fixed in 2.5% glutaraldehyde for 2-4 hours at 4°C, post-fixed in osmium tetroxide then pelleted in agar and processed for embedding in "Araldite" epoxy resin. 1µm thick sections of the cell pellet were examined to find areas with a high cell density which were then ultra thin sectioned. The 100nm sections were negatively stained and examined by transmission E.M. to look for cells with phagosomes containing bacteria and lysosome marker.

2.2.8. SDS-PAGE AND WESTERN BLOTTING

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in gradient 10-20% acrylamide vertical slab gels using the Laemmli (1970) buffer system. Pre-cast 8-25% gels were also used with the Pharmacia Excel gel and Phast horizontal electrophoresis systems. Samples were dissociated at 100°C

for 5 min in 0.0625M Tris HCl buffer pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue. In some cases 2-mercaptoethanol was omitted to look for proteins containing disulphide bonds. Protein loading on the slab gels was 25-30 μ g, 10-20 μ g was loaded on Excel gels and 1 μ g on the Phast gels. Molecular weight markers were Sigma, Dalton VII-L (14,200, 20,100, 24,000, 29,000, 36,000, 45,000 and 66,000 Da). Electrophoresis was at 200V constant voltage over 20h for vertical gels, 45mA constant current over 2-3 hours for Excel gels and 250v, 13mA for 40 min with Phast gels. Gels were stained with Coomassie blue or a silver stain for proteins (Bio-Rad).

Proteins in unstained gels were transferred onto nitrocellulose using a semi-dry electro-blotter (Bio-Rad) at 3mA/cm² of gel for 30 min. Following incubation with 3% gelatin in 50mM Tris buffered saline (TBS) pH 7.4 containing 0.05% Tween 20 (TTBS) the nitrocellulose was stained immediately or stored at -20°C. These blots were immunostained using a procedure described by Newell (1987). Following incubation with antisera, bound antibody was recognised with anti-rabbit or anti-guinea pig IgG conjugated to Horseradish peroxidase (HRP) which was visualised with 3-amino 9-ethyl carbazole as substrate.

2.2.9 ANTISERA

Antisera were raised in rabbits to whole, live, virulent and avirulent strains and to surface protein preparations. Rabbits were immunised as described by Conlan & Ashworth (1986).

Guinea pig anti-*L.pneumophila* antibodies were obtained from animals which had received sub-lethal doses of virulent strains and non-lethal high doses of avirulent strains.

Development of antibody by the animals was monitored by ELISA and immunoblotting techniques.

Antiserum to *L.micdadei* macrophage infectivity potentiator (mip, Cianciatto *et al.*, 1989b, Bangsberg *et al.*, 1991) was a generous gift from Dr. Jette Bangsberg, Statens Seruminstitut, Copenhagen, DK.

2.2.10. ABSORPTION OF ANTISERA

An adsorption technique was used to remove cross-reacting antibodies from certain of the antisera. A working dilution of the antiserum was incubated with an appropriate bacterial strain at a concentration of 10^{11} organisms for 1 hour at 37°C with constant rotation then stood overnight at 4°C. Following centrifugation to remove bacteria the antiserum was absorbed twice more. The efficiency of removal of antibodies was assessed by ELISA.

2.2.11. ELISA

The ELISAs used to screen for specific antibody production were similar to that described by Conlan & Ashworth (1986) where antigen was coated on microtitre plates and antigen-specific antibody detected by anti-rabbit or guinea pig IgG conjugated to Horseradish peroxidase (HRP) using tetramethyl benzidine as substrate.

2.2.12. LPS PREPARATIONS

Virulent and avirulent strains grown in YEB were harvested and a hot saline extraction of LPS prepared by the method of Conlan and Ashworth (1986). The extracts were separated on linear 15% acrylamide gels containing 4M urea and stained for LPS by the Tsai and Frasch (1982) silver stain.

2.2.13. EXTRACELLULAR ENZYME ASSAYS

Extracellular enzyme production by virulent and avirulent strains was compared by analysis of the supernatant following 18 hour culture in YEB and also by growth of strains on agar into which enzyme substrates were incorporated.

Tissue destructive protease (TDP) (Conlan *et al.*, 1986) activity was identified by casein precipitation whilst chromogenic p-nitroanilide substrates were used to show activities of leucine, lysine and alanine amino peptidases, acid phosphatase (Conlan *et al.*, 1986) and phospholipase C (Baine, 1985).

2.2.14. SURFACE STRUCTURE ANALYSIS

Colonies of virulent and avirulent strains were scraped from BCYE agar following 3 days incubation at 37°C and suspended in distilled water. The suspensions were vortexed for 15 seconds three times to shear off any loosely bound material. Bacteria were removed by centrifugation followed by passage through a 0.45µm pore diameter filter. The filtrate was concentrated by freeze-drying or by ultra centrifugation at 100,000g and analysed by SDS-PAGE and electron microscopy. Rabbit antiserum to this material was raised and used for immunolabelling techniques.

2.2.15. PREPARATION OF CELL WALLS

Whole bacteria were scraped from BCYE plates following 3 day incubation and suspended in HEPES buffer pH 7. Suspensions containing approximately 10^{10} - 10^{11} organisms ml⁻¹ were subjected to repeated bursts of 20,000 lbs/sq inch pressure in a French pressure cell. Broken bacterial suspensions were centrifuged to remove debris and the cell wall material pelleted in an ultracentrifuge at 100,000g. The cell

walls were washed in HEPES, then treated with 2% sarkosyl in HEPES containing 7mM EDTA at 37°C for 1h with vigorous mixing every 15 min. The insoluble outer cell wall material was pelleted from the soluble inner membrane by centrifugation and the detergent removed from the soluble material by dialysis.

The cell walls and inner cell membranes of virulent and avirulent strains were analysed by SDS-PAGE following each of the above procedures.

2.2.16. IMMUNOCYTOCHEMISTRY

Immunolabelling of antigens by electron and light microscopy was performed as described by Williams *et al.* (1987). Immunogold labelling was used to identify surface antigens on whole organisms taken from BCYE agar and an indirect immunoperoxidase technique used to investigate *in vivo* expression of these antigens in sections of lungs from guinea pigs with experimental LD.

2.2.17. EXTRACTION OF PROTEINS BY DETERGENTS

Outer membrane preparations and whole bacteria were treated with the following detergents: MEGA-10 (decanoyl-N-methylglucamide); MEGA-8 (Octanoyl-N-methylglucamide); n-Octylglucoside; Thesit (dodecylpoly-(ethyleneglycolether)_n); CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulphonate); CHAPSO (3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane-sulphonate).

Solutions (10% w/v) of the detergents were prepared in 50mM phosphate buffer containing 7mM EDTA. 20µl of detergent solution was added to 1ml of bacterial suspension (approximately 10¹⁰ cells ml⁻¹) and to 1ml of outer membrane protein preparations (approximately 2mg ml⁻¹). Following incubation at 37°C for 1 hour the detergent extracts were separated from the insoluble pellets and both analysed in gradient Excel and Phast gels.

2.3. RESULTS

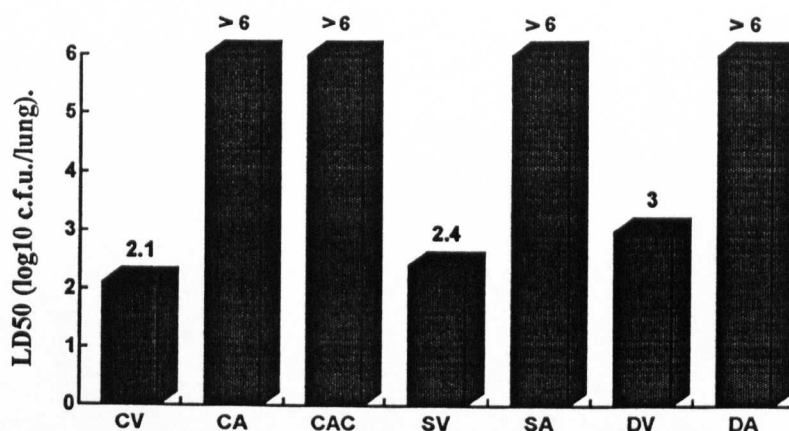
2.3.1. VIRULENCE OF STRAINS

Virulent strains Corby (CV), Stafford (SV) and Dodge (DV) and their avirulent counterparts (CA, SA and DA) were given to guinea pigs as a small particle aerosol to determine their virulence in terms of ability to cause disease and death. The LD₅₀'s and survival rates of the animals are given in Fig.2.2. Virulent strains caused illness typical of LD in the guinea pig with death occurring between 3-4 days whereas the derived avirulent strain was not lethal for the animals even when administered at the maximum dose possible (hence the LD₅₀ value is expressed as being greater than 1×10^6 c.f.u./ lung).

After 32 passages on BCYE the Corby strain became completely avirulent (CAC) in the guinea pig model (Jepras *et al.*, 1985). Stafford and Dodge strains did not convert to avirulent forms after subculture 94 times on BCYE (Slayne and Fitzgeorge, unpublished) but were avirulent after single passage on MHIH.

Once passaged the avirulent strains were able to grow on MHIH but virulent parent strains and CAC did not.

Fig.2.2. LD₅₀ values for *L.pneumophila* strains in guinea pigs infected by the aerosol route.



2.3.2. UPTAKE AND INTRACELLULAR MULTIPLICATION OF STRAINS BY MACROPHAGES *IN VITRO*.

The *in vitro* guinea pig macrophage assay was used to compare uptake and intracellular multiplication of the virulent and avirulent forms of the Corby strain.

Uptake of strains by macrophages from 20-120 min was similar for each of the strains in any one experiment. Fig.2.3. shows the results of three assays where strains were taken up to the same extent over 120 min in each experiment although there were variations, between assays, in the numbers of bacteria ingested over the time period.

Fig 2.4. shows the change in numbers of intracellular organisms over 48h. CV multiplied by 2 log₁₀ c.f.u. greater than the 0h count, CA increased less than one log₁₀ c.f.u. and CAC decreased by 3 logs.

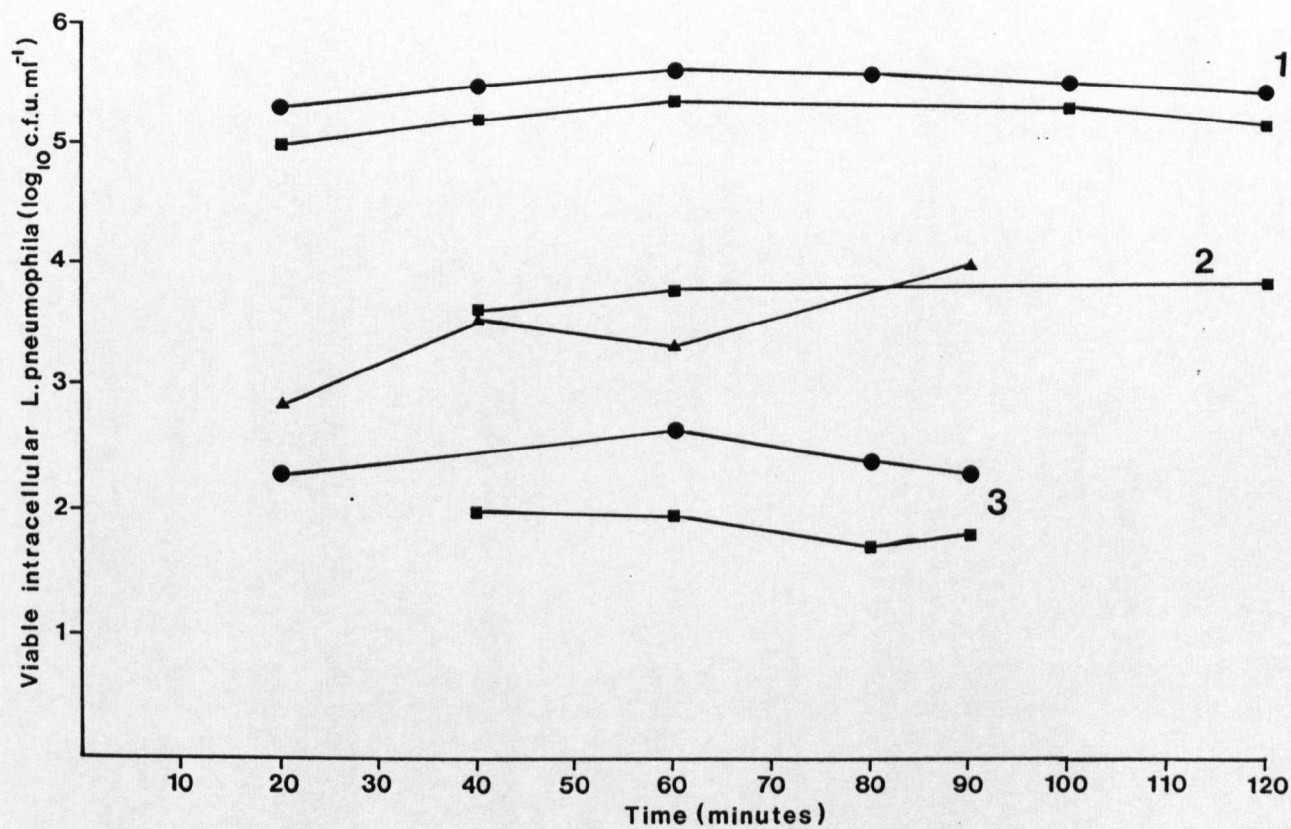


Fig.2.3. Uptake of Corby strains by guinea pig macrophages *in vitro*. Three experiments are shown, marked 1, 2, and 3.

■ - CV, ● - CA, ▲ - CAC.

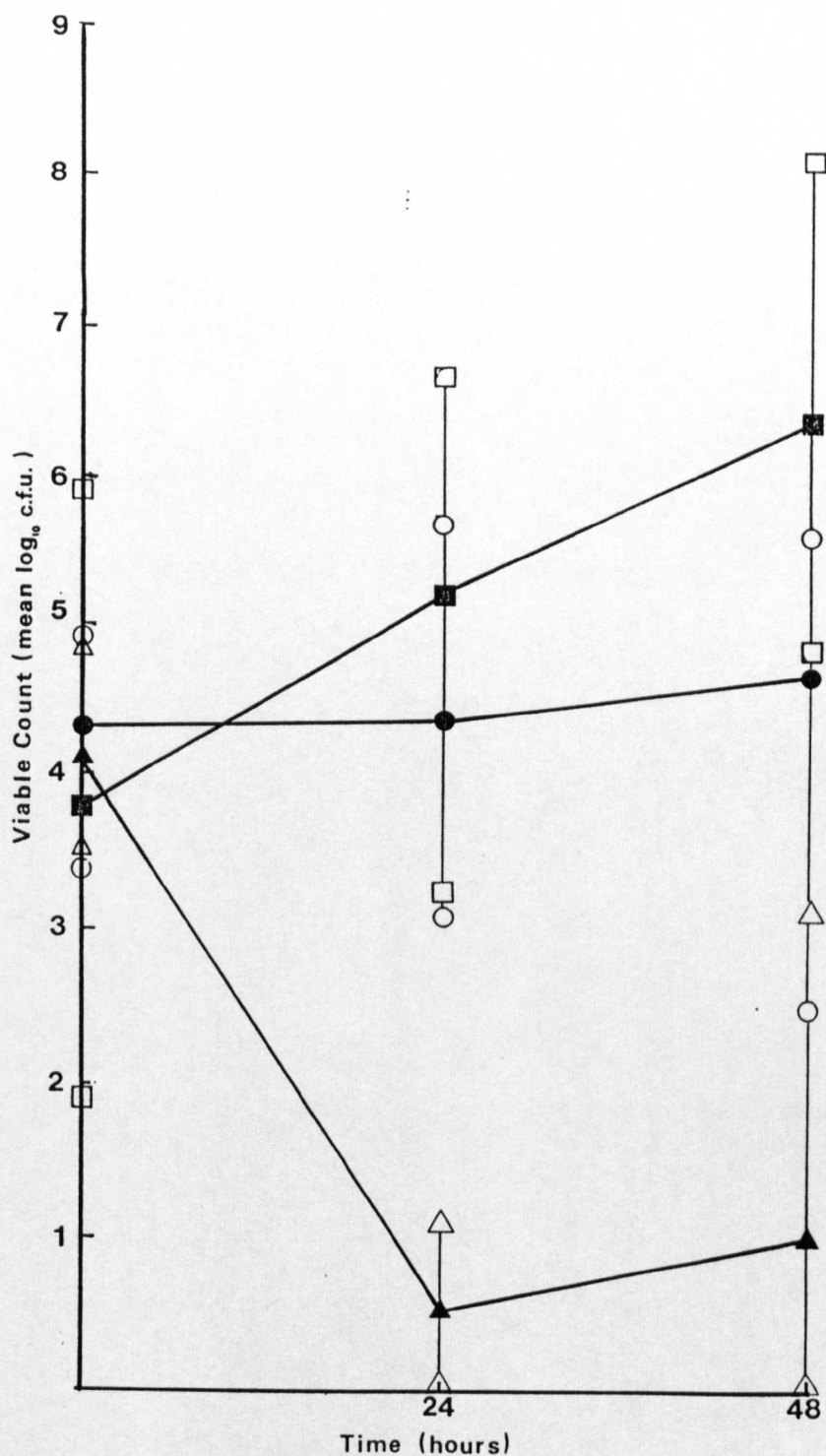


Fig.2.4 Intracellular multiplication of Corby strains in guinea pig macrophages maintained in cell culture over 48 hours. Solid symbols represent mean values of experiments, open symbols represent range of values.

■ - CV, ● - CA ▲ - CAC.

2.3.3. GROWTH OF STRAINS IN GUINEA PIG LUNG LAVAGE

Viable counts of CV and CA in cell-free *L.pneumophila*-infected and uninfected guinea pig lung lavages are shown in Fig.2.5. When compared with growth in YEB both strains exhibited little or no multiplication.

The virulent bacteria lost viability in the various lavages over 24 hours and were undetectable after 48 hours suggesting that the lavages were inhibitory. However in a second experiment viability was maintained in normal lung lavage over 48 hours. Viability of avirulent Corby decreased slightly over 48 hours in normal lavage and increased slightly in infected lavage. Lavages which had been concentrated (as described in the methods section) had similar effects on the growth of CV and CA as above. Addition of ferric pyrophosphate to the lavages did not alter the results (data not shown). In general, survival of the avirulent bacteria was better than the virulent bacteria.

2.3.4. PHAGOSOME LYSOSOME FUSION

Labelling of lysosomes was not successful with either Thoria sol or Colloidal gold. The Thoria sol was not confined to the lysosomes and was too amorphous to be clearly distinguishable from components of the cytoplasm. The colloidal gold could not be seen in the lysosomes. Macrophages examined by electron microscopy 24 hours after infection with CV in tissue culture revealed that lysosomes in the vicinity of *Legionella*-filled phagosomes appeared to be discharging their contents into the phagosome via a disintegrating membrane. A typical example is shown in Fig.2.6.

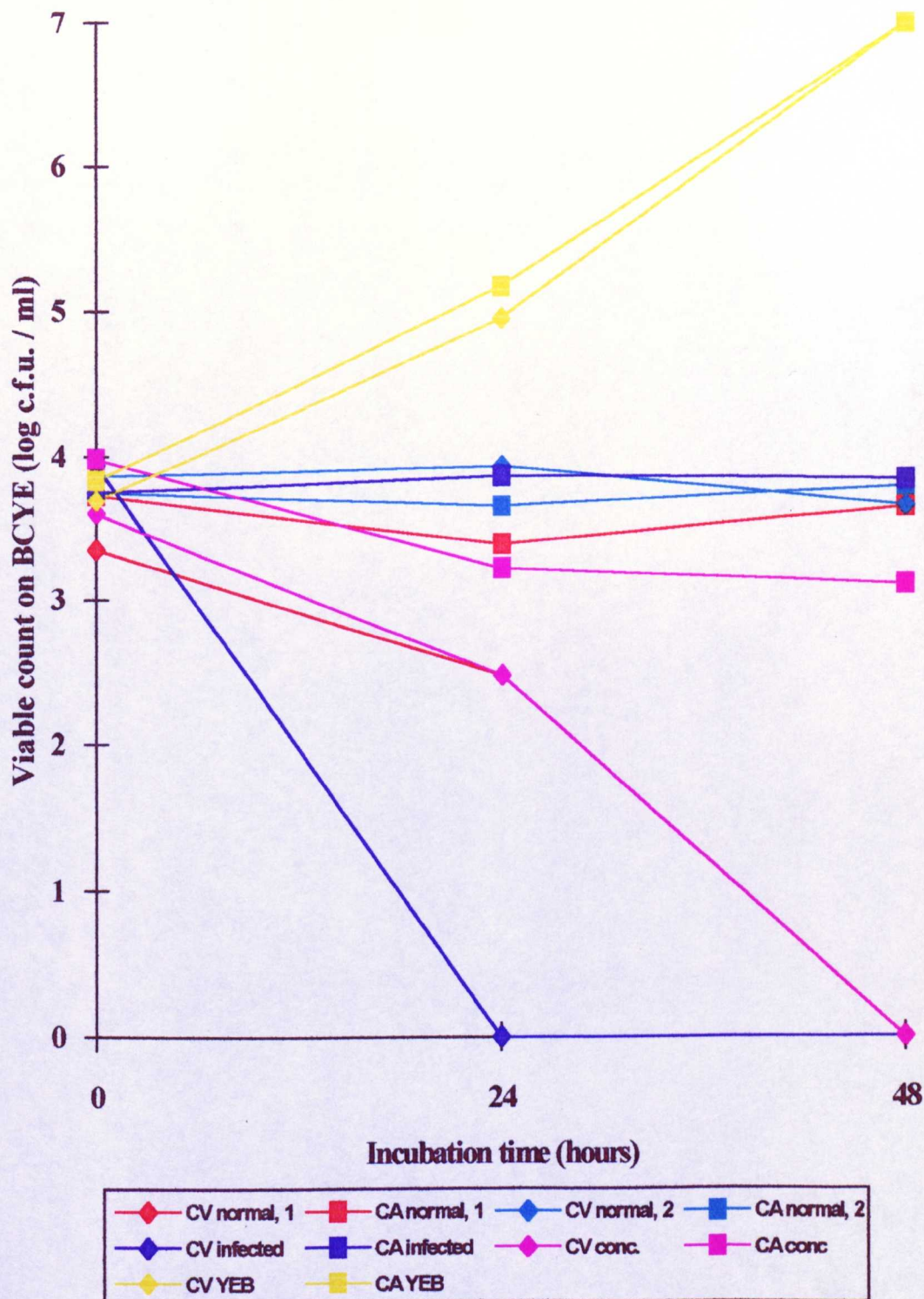
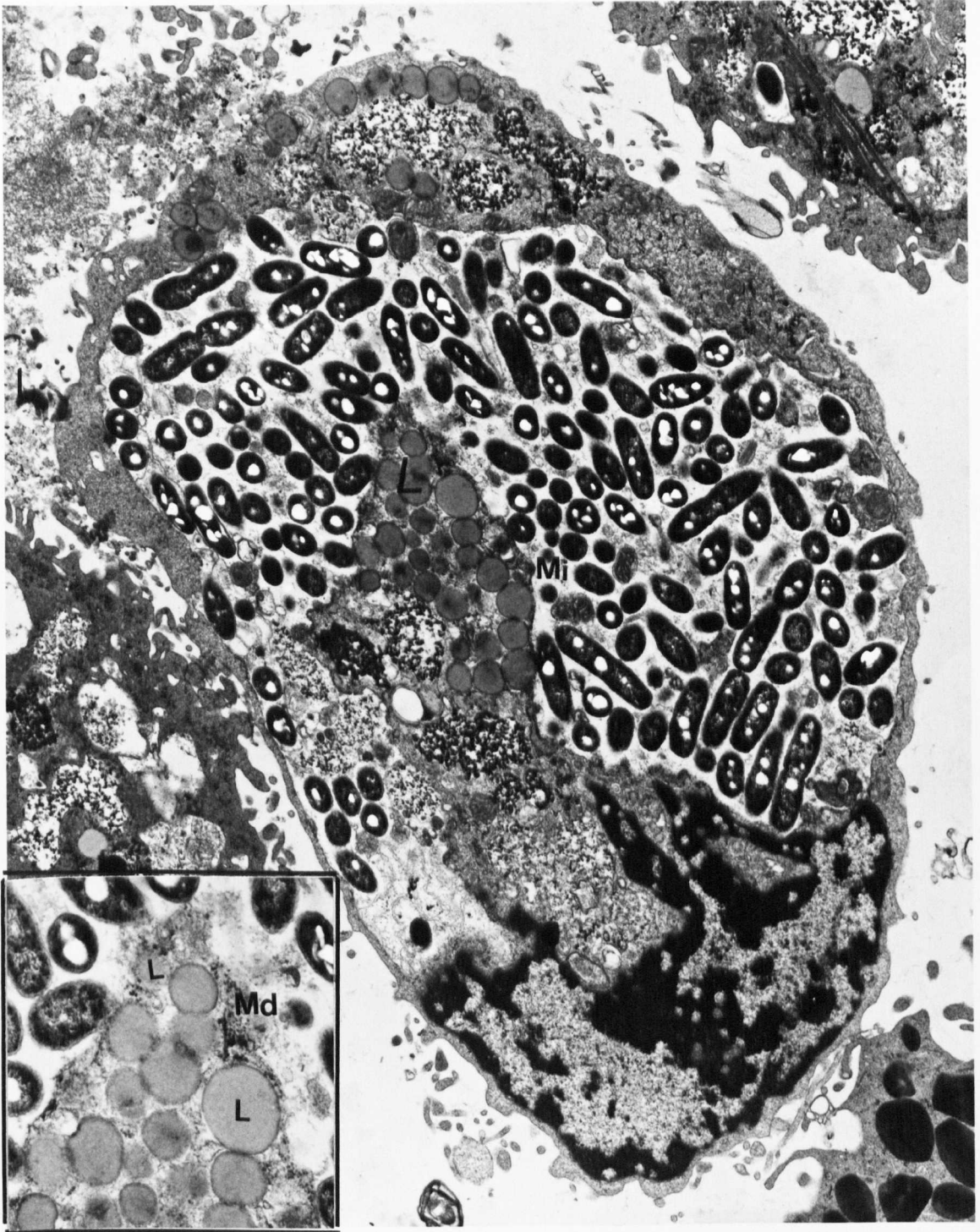


Fig.2.5. Survival of Corby strains in cell-free lung lavages of infected and uninfected guinea pigs. Log₁₀ viable counts are the mean of 3 tests. Two experiments with normal lavage fluid are shown and one experiment with infected lavage and concentrated, infected lavage.

Fig.2.6. Electron micrograph of a guinea pig macrophage 24 hours after infection with virulent *L.pneumophila* showing lysosomes (L) fusing with a *Legionella*-filled phagosome (x 10,000). Insert is the area at higher magnification (x19,000) to show disintegration of phagosome membrane (Md) as compared with an intact membrane (Mi, centre, main picture).



2.3.5. LPS

Crude preparations of LPS were prepared and analysed by SDS-PAGE on a 15% gel containing urea. This silver-stained gel is shown in Fig.2.7. The ladder banding pattern typical of *L.pneumophila* LPS serotype 1 was seen in all the strains and there was little difference between virulent and avirulent strains.

2.3.6. ENZYME ASSAYS

The extracellular enzyme activities of the three pairs of virulent and MHIH avirulent strains were compared. Table 2.1 shows that all strains (when grown on ABCD agar or in YEB) had the same enzyme activities qualitatively.

Table 2.1. Extracellular enzyme assays of virulent and avirulent strains

	STRAIN						
	CV	CA	CAC	SV	SA	DV	DA
Enzyme substrate	√	√	√	√	√	√	√
Casein	√	√	√	√	√	√	√
Leucine pNA	√	√	√	√	√	√	√
Lysine pNA	√	√	√	√	√	√	√
Alanine pNA	√	√	√	√	√	√	√
pNA phosphate	√	√	√	√	√	√	√
phosphoryl choline pNA	√	√	NT	√	√	√	√

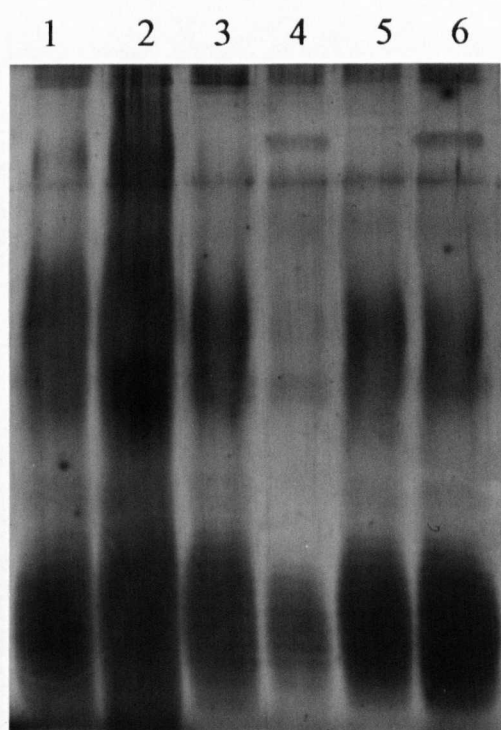


Fig.2.7. SDS-PAGE analysis of virulent and avirulent strain crude LPS preparations in a 15% acrylamide gel containing 4M urea.

- 1. CV;
- 2. CA;
- 3. SV;
- 4. SA;
- 5. DV;
- 6. DA.

2.3.7 SURFACE PROTEINS

The surface material obtained by vortexing whole organisms was analysed by SDS-PAGE (Fig.2.8). A 45kDa protein band was found to be present in much greater quantity on virulent organisms than avirulent. When examined by electron microscopy this material consisted mainly of flagella, fimbriae and a lattice-type cell wall structure (Fig.2.9).

Antisera raised against the flagellar material contained anti LPS antibodies as determined by ELISA and also, on Western blots, reacted strongly with the 45kDa band. To determine whether this band was flagellin the antisera were adsorbed with CA (which contains few flagella) to remove anti LPS antibodies. Fig.2.10, shows that the antiserum no longer reacts with LPS or CA flagella preparation but stains the 45kDa band strongly. The adsorbed anti-flagella antiserum was used to immunogold label whole bacteria and flagella preparations which were then examined by transmission electron microscopy. Fig 2.11a shows whole CV with labelled flagella, 2.11b shows a SV surface protein preparation with flagella and unlabelled fimbriae and 2.11c shows a flagella preparation from SA showing that the avirulent strains do possess some flagella.

When used to immunoperoxidase label infected guinea pig lung tissue the adsorbed anti-flagella antibody produced strong staining in the areas of tissue damage which, in sections stained for LPS, contained most of the bacteria (Fig.2.12).

2.8. OUTER MEMBRANE ANALYSIS

Outer membrane preparations (OMPs) of the pairs of strains following sarkosyl extraction gave variable results on SDS-PAGE. Fig 2.13a shows a gel of OMPs made from one batch of bacteria in which a 24kDa band

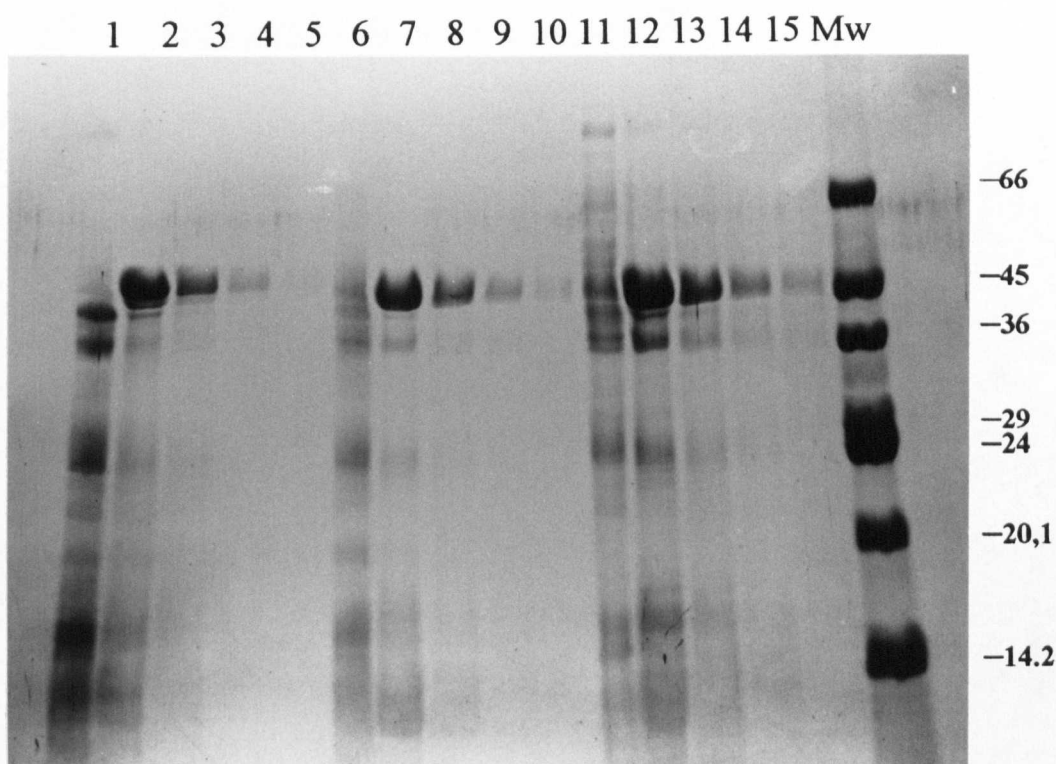


Fig.2.8. SDS-PAGE of surface proteins of virulent and avirulent strains.

- | | | |
|--------------------|--------------------|---------------------|
| 1. CA; | 6. SA; | 11. DA; |
| 2. CV; | 7. SV; | 12. DV; |
| 3. CV diluted 1/2; | 8. SV diluted 1/2; | 13. DV diluted 1/2; |
| 4. CV, 1/4; | 9. SV, 1/4; | 14. DV, 1/4; |
| 5. CV, 1/8; | 10. SV, 1/8; | 15. DV, 1/8; |

Mw, molecular weight markers as shown.

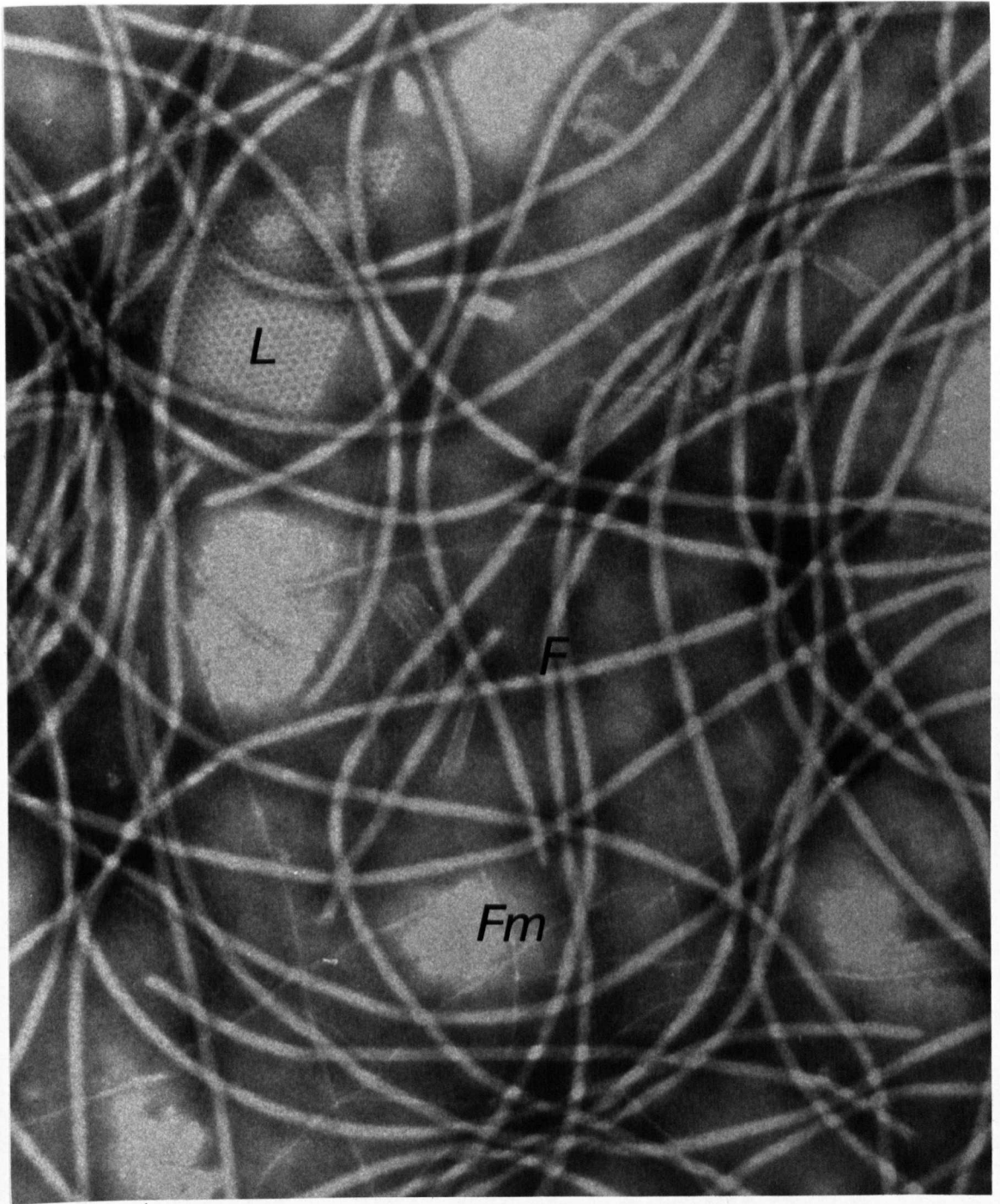


Fig.2.9. Electron micrograph of surface proteins from SV. Flagella (F), fimbriae (Fm) and a lattice type structure (L) were observed.(x 142,000)

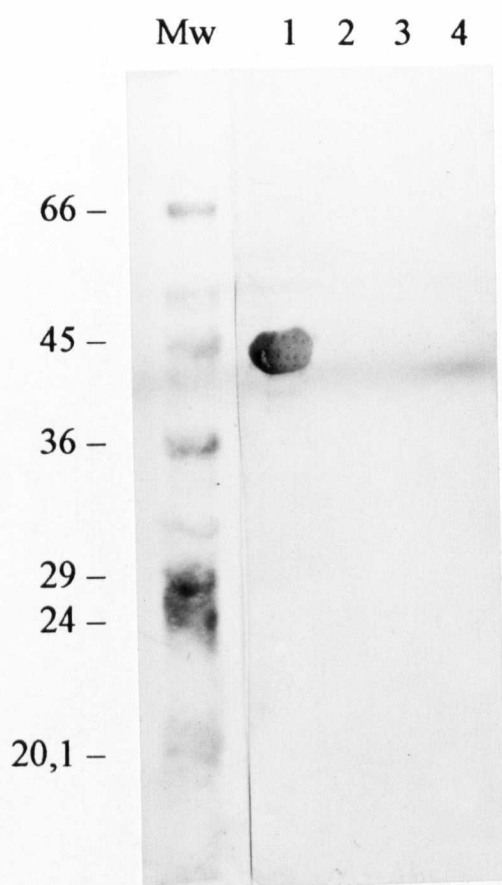


Fig.2.10. Western blot of CV and CA surface protein and LPS preparations immunostained with antiserum raised against virulent strain surface proteins and adsorbed with whole avirulent strains.

1. CV surface protein prep.;
2. CA surface protein prep.;
3. CV LPS;
4. CA LPS.

Molecular weight markers (Mw) stained with colloidal gold total protein stain.

A

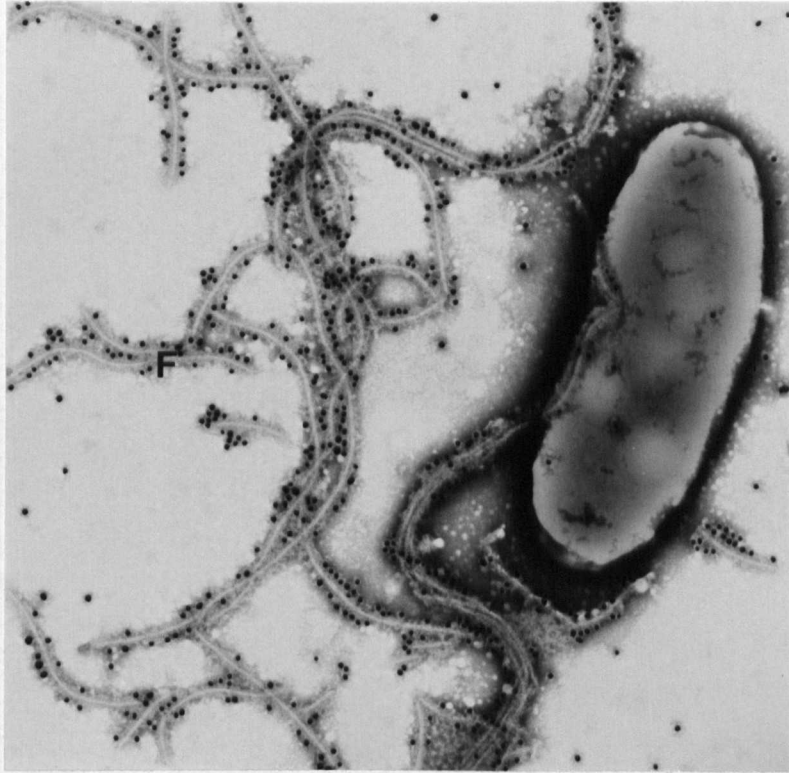
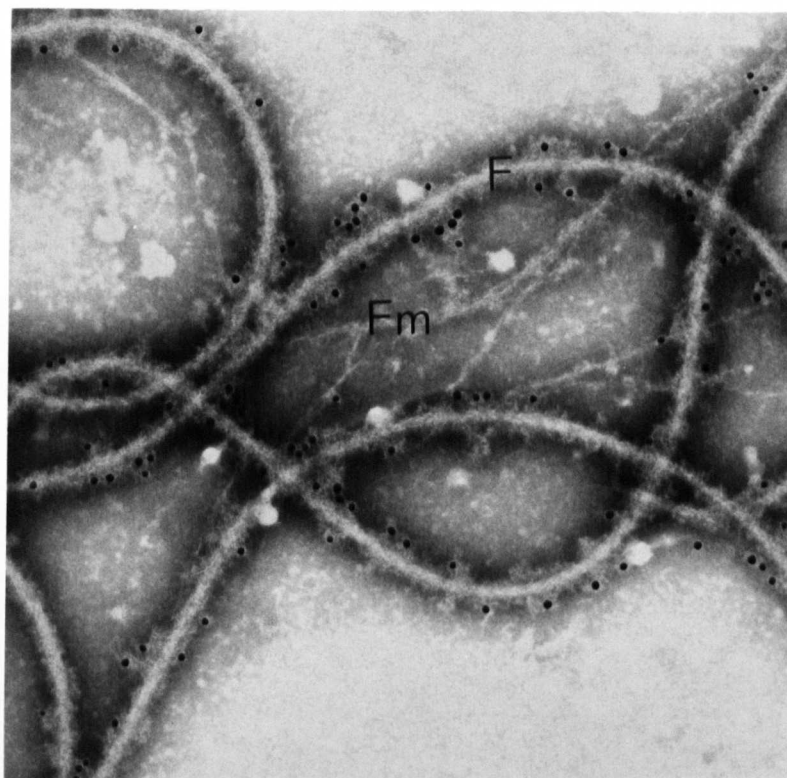


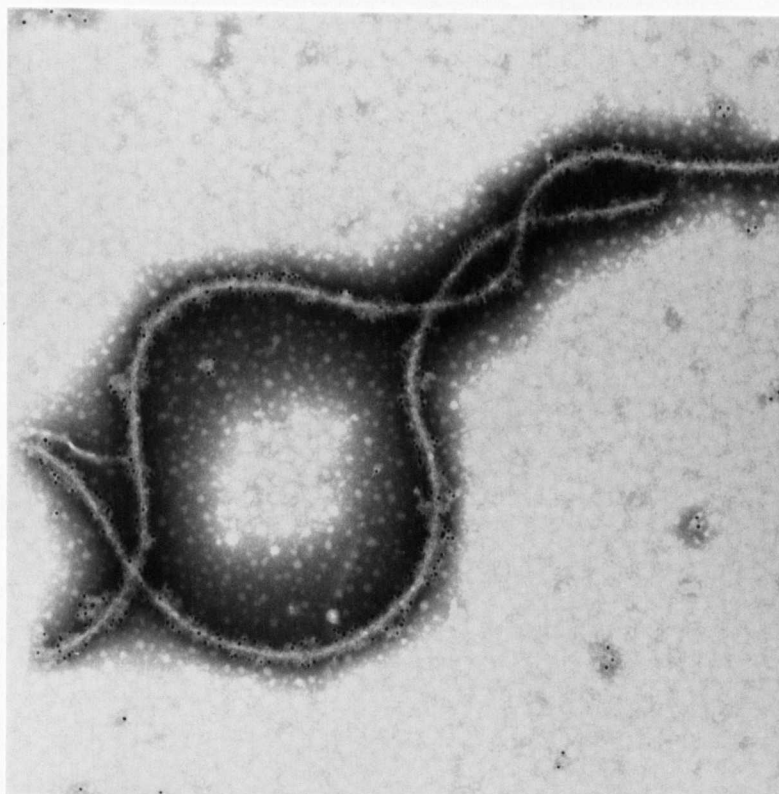
Fig.2.11 Electron micrographs of whole bacteria and surface protein preps. of virulent and avirulent strains immunogold labelled with adsorbed anti-surface protein antibodies. The gold particles attached only to flagella (F) and not to whole bacteria or fimbriae (Fm).

A. CV whole cells, x 40,000;

B



C



B. SV surface protein prep x 100,000.;

C. SA surface protein prep x 50,000.

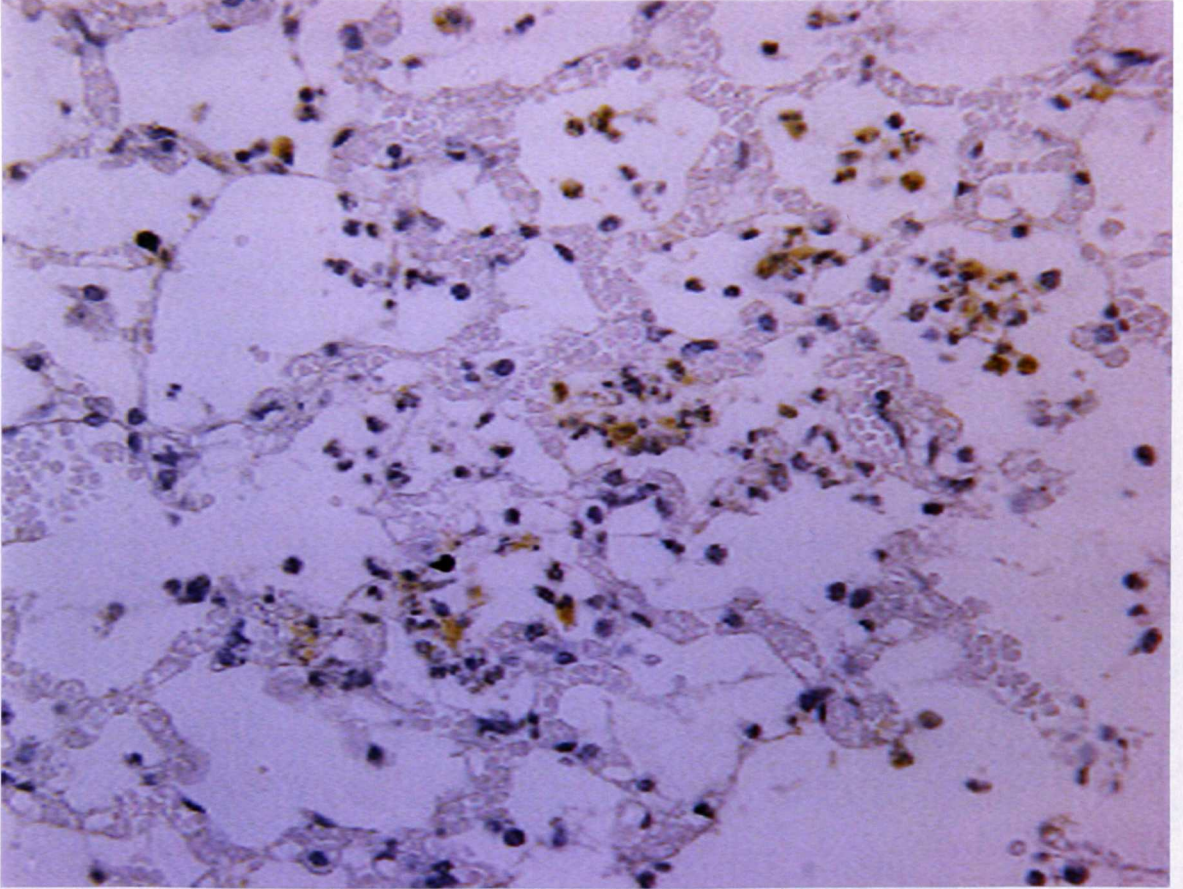


Fig.2.12. Micrograph of a section of CV-infected guinea pig lung, immunoperoxidase stained for flagella using serum absorbed to remove antibodies against LPS. The brown areas represent labelled flagella. Tissue counterstained with haematoxylin. x500 magnification.

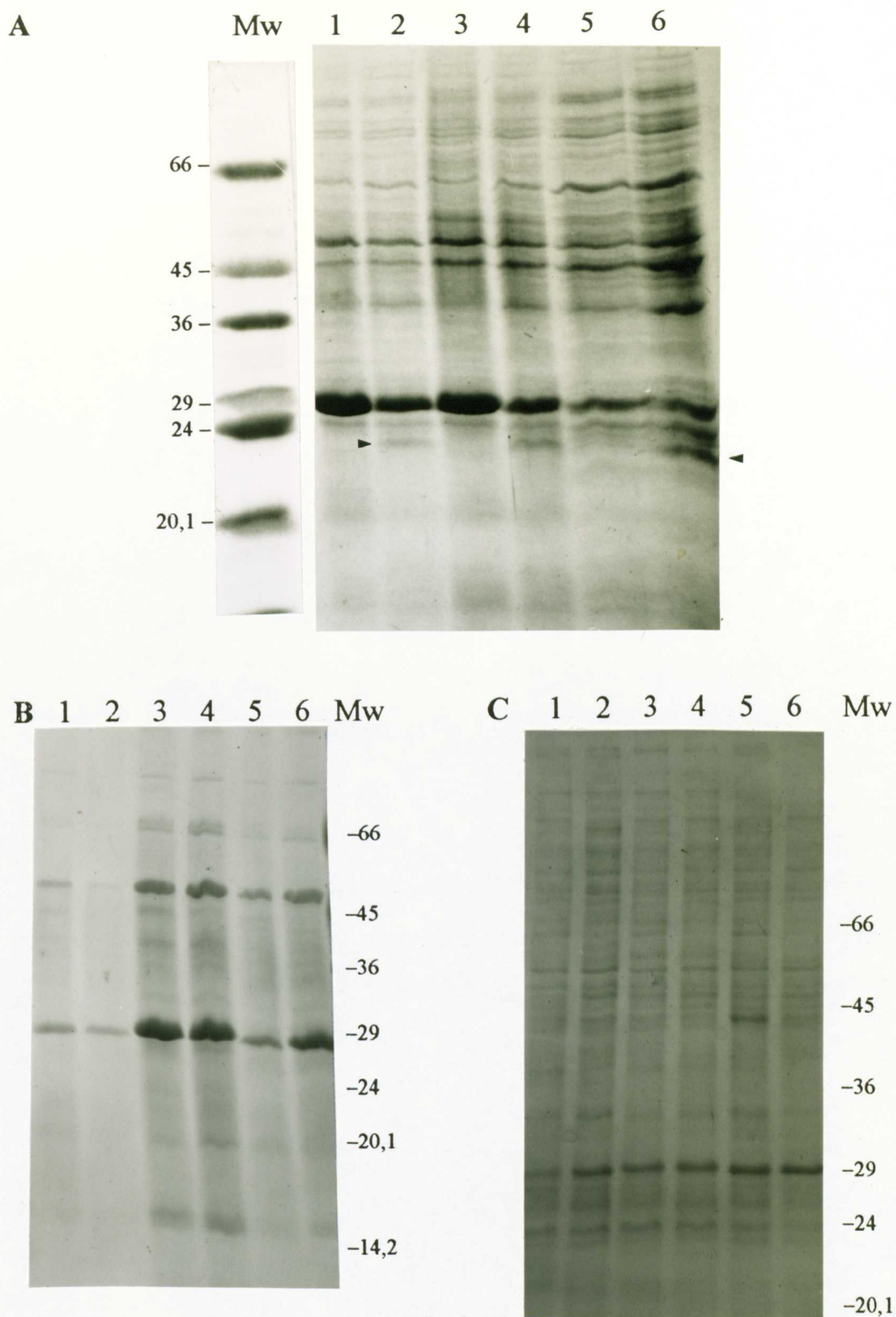


Fig.2.13. SDS-PAGE of outer cell walls and inner membranes of virulent and avirulent Corby, Stafford and Dodge strains. Arrow marks virulent strain-specific band. A and B, Outer cell walls, C, Inner cell membranes.

Lanes on A: 1. CA, 2. CV, 3. SA, 4. SV, 5. DA, 6. DV.

Lanes on B and C: 1. CV, 2. CA, 3. SV, 4. SA, 5. DV, 6. DA.

(arrowed) was present in the virulent strains but not the avirulent. However Fig.2.13.b shows OMPs which do not have this difference and is more typical of subsequent preparations.

The sarkosyl solubilised inner membrane proteins are shown in Fig.2.13c, these also exhibited much similarity between virulent and avirulent strains. Fig.2.14. is a gel of outer membrane preparations run under reducing and non-reducing conditions. There were no differences between virulent and avirulent strains but the arrows represent proteins seen with reduced samples which were not present in the non-reduced samples.

2.3.9. IMMUNOSTAINING OF WHOLE ORGANISM AND OUTER MEMBRANE PROTEIN BLOTS

Western blots of whole organisms and outer membrane preparations of virulent and avirulent strains were stained with various antisera.

A) Reaction with monoclonal antibody 2.

Both virulent and avirulent strains reacted with a monoclonal antibody which has been reported to react with clinical but not environmental strains of *L.pneumophila* (MAb 2, Dournon *et al.*, 1986) by immunofluoresence (not shown) and by immunoblotting. Fig.2.15 is a representative blot of the pairs of strains demonstrating an identical banding pattern for CV as CA.

B) Polyclonal rabbit sera and sera from infected guinea pigs.

Similar staining patterns of protein bands were observed on western blots of SV and SA whole bacteria when incubated with sera from rabbits immunised with either whole SV or SA (Fig.2.16 lanes 1 & 2, 3 & 4). There was however a slight difference in the intensity of staining of a protein band in the region of the 24 kDa marker in that it was stronger in the SV blots. When the SV and SA blots were incubated with sera from guinea pigs which had been infected with SV there were fewer reactions

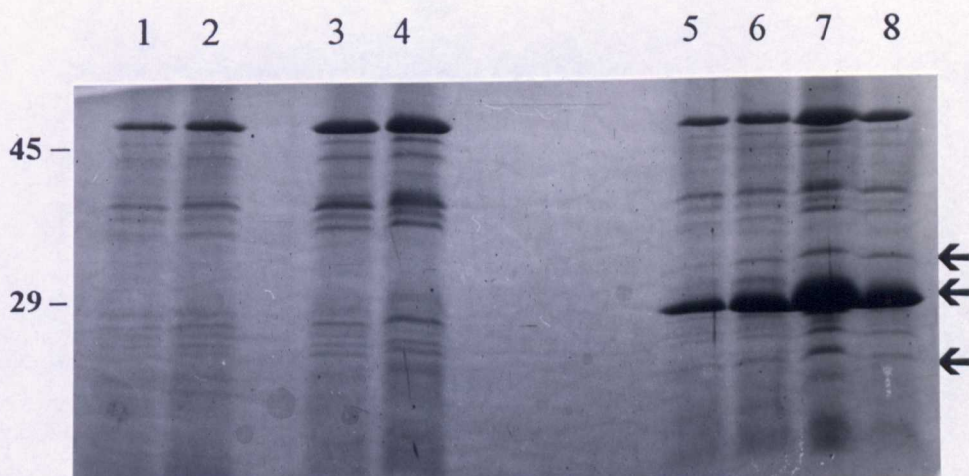


Fig.2.14. SDS-PAGE of outer membrane preps. of virulent and avirulent strains under non-reduced (lanes 1-4) and reduced conditions (lanes 5-8).

- | | |
|--------------------|----------------|
| 1. CV non-reduced; | 5. CV reduced; |
| 2. CA non-reduced; | 6. CA reduced; |
| 3. SV non-reduced; | 7. SA reduced; |
| 4. SA non-reduced; | 8. SV reduced. |

Position of molecular weight markers of 45 and 29 kDa are shown. Protein bands found only under reduced conditions are marked (arrows).

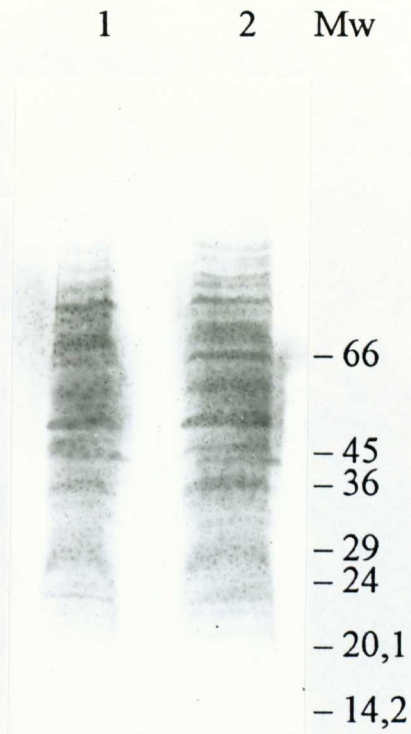


Fig.2.15. Western blot of Corby virulent (lane 1) and avirulent (lane 2) outer membrane preps immunostained with monoclonal antibody 2 (MAb 2) showing identical band patterns.

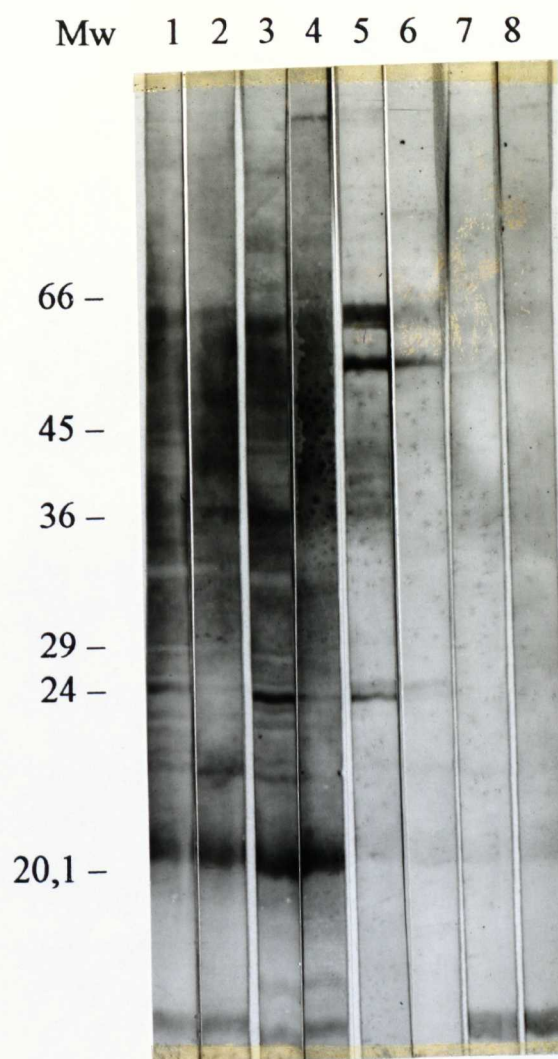


Fig.2.16. Western blot of Stafford strain whole bacteria immunostained with various anti-*L.pneumophila* sera. Odd numbered lanes are virulent bacteria (SV) and even numbered lanes are avirulent (SA).

Antisera:

lanes 1 & 2, rabbit anti SV whole bacteria;

3 & 4, rabbit anti-SA whole bacteria;

5 & 6, serum from guinea pigs infected with SV;

7 & 8, serum from guinea pigs infected with SA.

than those observed with the rabbit sera and even fewer when stained with sera from guinea pigs infected with SA.

Fig.2.17 shows the results of staining Corby OMP blots. Staining patterns of the protein bands were similar on the CV and CA blots following incubation with rabbit sera. When the same blots were incubated with sera from guinea pigs infected with CV and CA the intensity of staining was less than with the rabbit sera but most of the protein bands which had stained with the rabbit sera, were also observed with the guinea pig sera. A high molecular weight band was observed only on CA blots (arrow).

C) Anti-mip antibody

Western blots of whole bacteria and outer membrane preparations of the Corby virulent and avirulent forms all showed positive reactions when incubated with rabbit anti-*L.micdadei* mip (Fig.2.18). The strongest reaction was with a band of around 24kDa but possible cross reactive bands at 29, 36, and 45kDa were also stained

2.3.10. DETERGENT EXTRACTIONS

The SDS-PAGE separation of proteins extracted from the membranes of whole CV and CA organisms by a number of detergents is shown in Fig.2.19. When incubated with either, phosphate buffer containing EDTA, the non-ionic detergent, octylglucoside (Fig.2.19a) or the zwitterionic CHAPS and CHAPSO (Fig.2.19b), the same protein bands were found in CV as CA and these were similar for each detergent. Extraction with the detergent, MEGA 10 resulted in a difference in protein bands between CV and CA (Fig.2.19c). A low Mw (<14kDa) was seen in greater quantity in CV than CA. This band was also prominent in the CV whole bacteria and when the pellets of the detergent

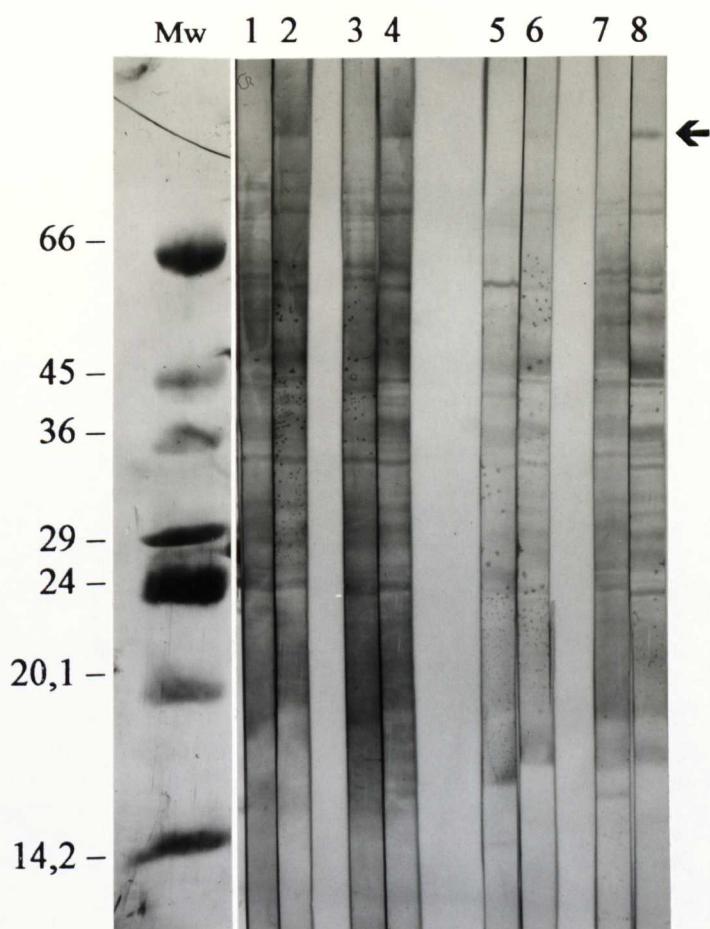


Fig.2.17. Western blot of Corby strain outer membrane proteins with various anti-*L.pneumophila* sera. Odd numbered lanes are a virulent strain preparation (CV) and even numbered lanes are avirulent (CA).

Antisera:

1 & 2, rabbit anti CV whole organisms;

3 & 4, rabbit anti-CA whole organisms;

5 & 6, serum from guinea pigs infected with CV;

7 & 8, serum from guinea pigs infected with CA.

Molecular weight markers (Mw) and CV stained with colloidal gold total protein stain. The arrow marks a possible avirulent strain specific band referred to in the text.

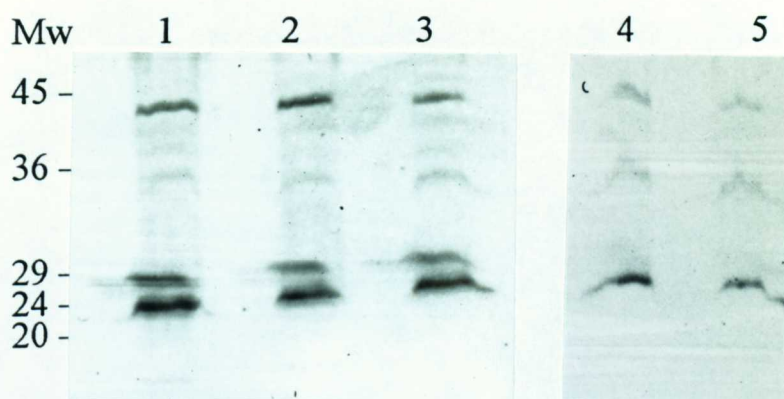


Fig. 2.18. Western blot of Corby strains immunostained with rabbit antiserum to *L.micdadei* m.i.p. protein.

Lanes: 1. CV whole bacteria;
 2. CA whole bacteria;
 3. CAC whole bacteria;
 4. CV outer membrane preparation;
 5. CA outer membrane preparation.

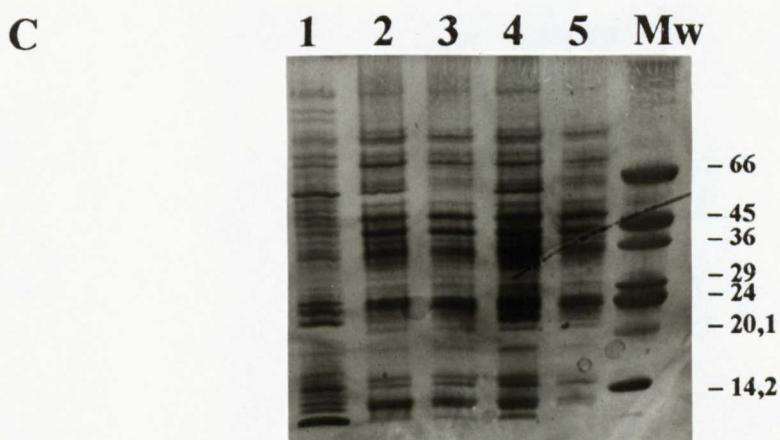
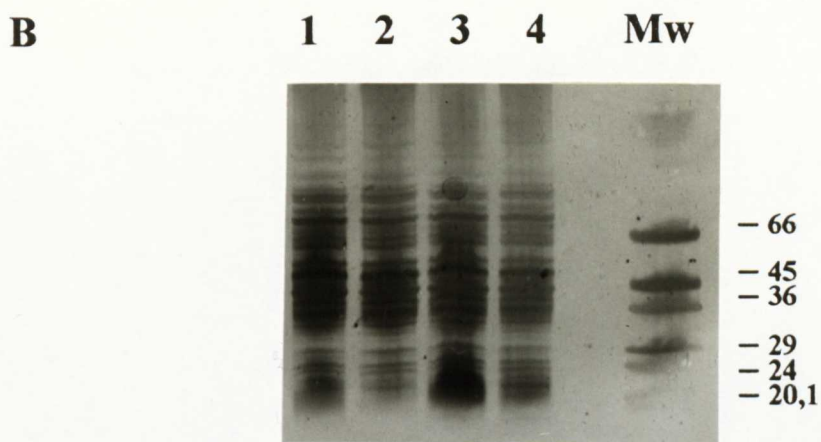
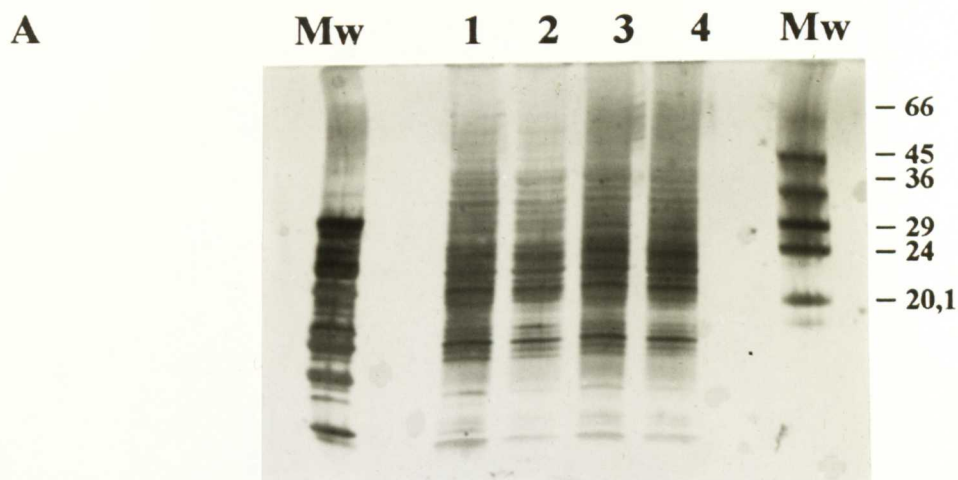


Fig.2.19. SDS-PAGE of detergent extracts of Corby virulent and avirulent strains. 8-25% Pre-cast gradient gels, silver stained.

A. 1. CV + EDTA buffer;
2. CA + EDTA buffer;
3. CV + octylglucoside;
4. CA + octylglucoside.

B. 1. CV + CHAPS;
2. CA + CHAPS
3. CV + CHAPSO;
4. CA + CHAPSO.

C. 1. CV whole organisms
2. CV + Mega 8
3. CA + Mega 8
4. CV + Mega 10;
5. CA + Mega 10.

extracted strains were analysed the band was present in all the CV but not CA (Fig.2.20a lanes 1-8, 2.20b lanes 8-17) samples and was absent in CA whole organisms (Fig. 2.20b lane 2). However, analysis of Stafford and Dodge showed a band at the same position on the gel in both virulent and avirulent whole organisms (Fig.20b lanes 3-6). CAC also contained the band (20b lane 7).

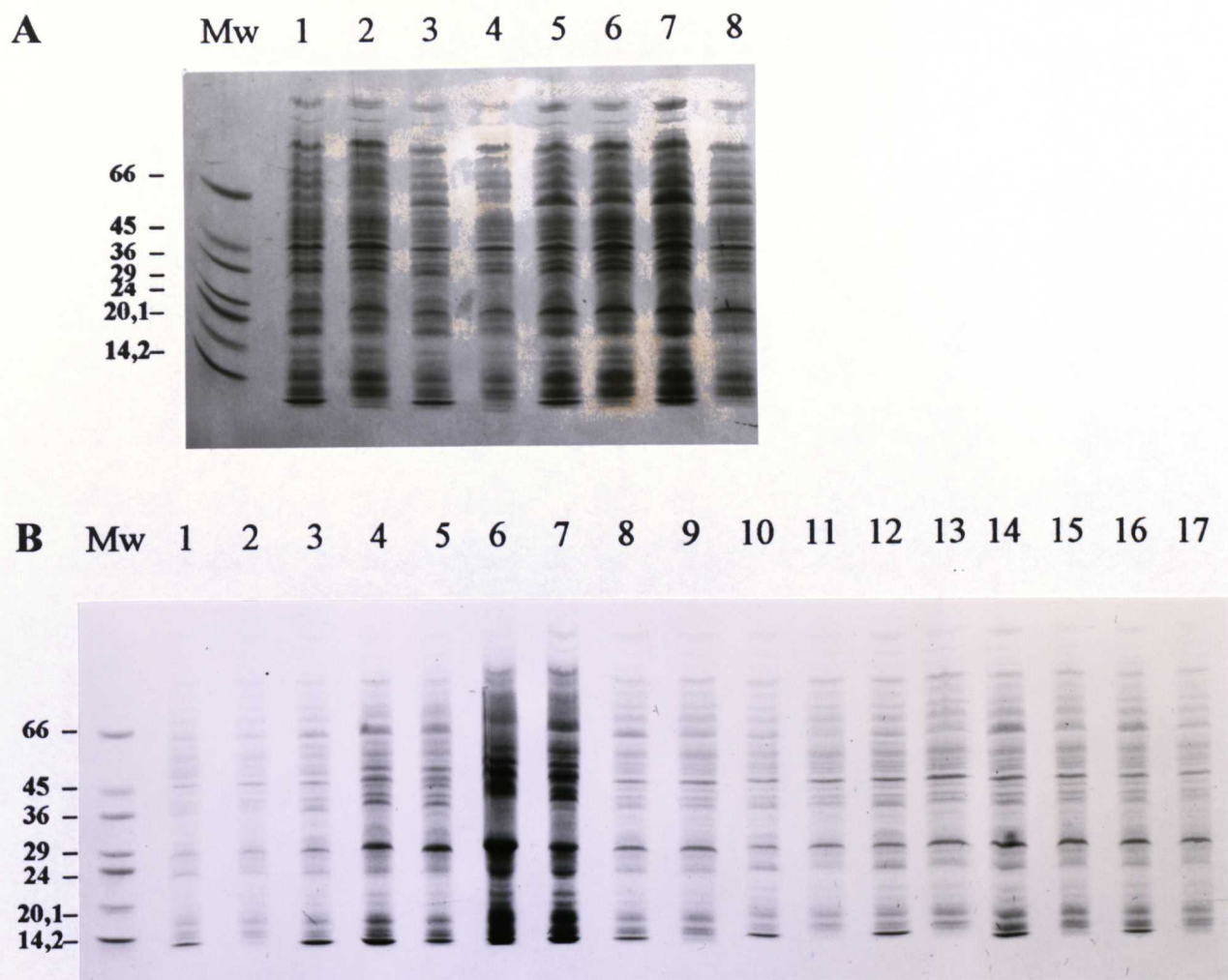


Fig.2.20. SDS-PAGE of the insoluble pellets of virulent and avirulent strain whole cells following incubation with various detergents 8-25% Pre-cast gradient gels, stained with Coomassie blue.

A. Lanes: 1,3 & 5. CV + Mega 8;

2,4 & 6. CA + Mega 8;

7. CV + Mega 10;

8. CA + Mega 10.

B Lanes: 1. CV control (no detergent);

2. CA control;

3. SV control;

4. SA control;

5. DV control;

6. DA control;

7. CAC control;

8. CV + octylglucoside;

9. CA + octylglucoside;

10. CV + CHAPS;

11. CA + CHAPS;

12. CV + CHAPSO;

13. CA + CHAPSO;

14 & 16. CV + buffer

15 & 17. CA + buffer.

2.4. DISCUSSION

The conversion of *L.pneumophila* to an avirulent form by subculture on Mueller Hinton agar (MHIH) was first documented by McDade and Shephard (1979) and since then several workers have investigated this phenomenon (Wong *et al*, 1981, Elliot and Johnson, 1982, Horwitz, 1987, Catrenich and Johnson, 1988) . In those studies virulence was assessed in embryonated hens eggs or guinea pig intraperitoneal or intratracheal infection models. Varying degrees of virulence attenuation were described for bacteria which had been passaged on MHIH at least 5 times. The results presented in this study demonstrate that highly virulent strains of *L.pneumophila*, following a single passage on MHIH, were completely avirulent in a guinea pig aerosol infection model of Legionnaires' disease. Virulent and avirulent forms of the Corby strain were incubated with guinea pig alveolar macrophages in tissue culture, and the virulent strain (CV) multiplied intracellularly over a 48 hour period, resulting in destruction of the cell monolayer. The MHIH-derived avirulent strain (CA) neither multiplied in nor affected the physical appearance of the macrophages. Another avirulent form of the Corby strain, derived from passage on BCYE agar (CAC), was also unable to multiply within macrophages but differed from CA in terms of its intracellular survival. The BCYE derived strain did not survive intracellularly but the MHIH avirulent strain remained viable over 48 hours. This may indicate that the inability of the avirulent strains to cause disease in guinea pigs was due to a susceptibility to different host defence mechanisms.

The *in vitro* assay suggested that guinea pig macrophages were unable to kill CA. If this is also the case in the lungs, it is possible that the mechanism of clearance is destruction by polymorphonuclear phagocytes (PMNs). An increased oxidative burst by PMNs in response to MHIH derived strains

compared with the parent virulent organism has been described (Summersgill *et al*, 1988). The virulent form of the Corby strain used in this study has been shown to be susceptible to PMNs *in vitro* (Jepras & Fitzgeorge, 1986) and *in vivo* (Fitzgeorge *et al*, 1988) and it is likely that the avirulent strains would also be readily killed by these cells. The fate of the Corby strains *in vivo* was studied in relation to lung phagocytes and is described in Chapter 3 of this thesis.

The avirulent form which had been attenuated on BCYE was unable to grow on MHH supporting the theory that attenuation on the two types of media resulted in different changes in each but the overall effect was that both were avirulent in guinea pigs.

The importance of intracellular growth for the pathogenesis of *L.pneumophila* was confirmed in these studies by an inability of any of the strains to grow in guinea pig lung lavage even when this was obtained from infected animals. This implies that the large numbers of extracellular bacteria seen in lungs of guinea pigs during experimental LD (Baskerville *et al.*, 1983, Williams *et al.*, 1987) had been released from macrophages.

The behaviour of MHH avirulent strains in the *in vitro* assay agree with those of Horwitz (1987) who characterised MHH mutants of the Philadelphia 1 strain and found that avirulent forms remained viable within a phagolysosome but were not able to multiply. Horwitz suggested that virulent strains inhibited phagosome-lysosome fusion thereby evading the bactericidal mechanisms of the macrophage but that the avirulent organisms, although not destroyed by the lysosome contents were unable to multiply. Rechnitzer & Blom (1989) have shown that phagosome lysosome fusion in human alveolar macrophages infected with *L.pneumophila* occurs whether the strains are virulent or not. It was not possible to determine whether the three forms of the Corby strain inhibited phagosome lysosome fusion in the early stages of infection but some evidence of fusion was found in electron

micrographs of macrophages 24 hours after infection with CV. This supports the findings of Rechnitzer & Blom, (1989) and suggests that strains which can multiply intracellularly are more likely to be resistant to the anti-microbial strategies of macrophages rather than just able to evade them..

There is evidence that both virulent and avirulent strains are highly susceptible to the toxic oxygen metabolites produced by stimulated phagocytes (Locksley *et al.*, 1982, Jepras and Fitzgeorge, 1986) however inhibition of the oxidative burst of phagocytic cells by virulent *L.pneumophila* has been described (Summersgill *et al.*, 1988 and 1990, Rechnitzer *et al.*, 1987) with virulent bacteria being slightly more inhibitory than avirulent. The oxygen-dependent mechanisms are not the only anti-microbial defences and if phagosome-lysosome fusion occurs the bacteria must also resist attack by lysosomal enzymes. *Leishmania amazonensis* amastigotes are able to resist oxygen-independent anti-microbial systems of macrophages and are able to grow in an environment of increased lysosomal enzyme activity (Prina *et al*, 1990). It is possible that this also applies to *L.pneumophila*. Virulent strains of *L.pneumophila* have been reported to inhibit acidification of phagosomes (Horwitz and Maxfield 1984) which may reduce activity of lysosomal enzymes, such as acid phosphatase, that require a low pH. It is not known if MHIH mutants inhibit acidification or lysosomal enzyme activity and assays to compare enzyme activity levels in CV and CA infected macrophages may reveal this. Relative susceptibilities of CA and CV to lysosomal enzymes such as cathepsins and dipeptidyl peptidases and to disrupted whole macrophages from normal or infected guinea pigs may reflect differences in intracellular growth. The MHIH avirulent forms must resist killing to a certain extent since viability is maintained within macrophages over 48 hours. Thus the key to whether these strains multiply or not could be

components of the lysosome to which virulent forms are resistant and avirulent forms are, at least partially, susceptible.

The reason for the loss of virulence of MHH strains was confirmed to be an inability to multiply within alveolar macrophages. To determine why this may be so, CV and CA were compared physically, biochemically, and immunologically in an attempt to identify a defect in some of the pathogenic and virulence factors which have been described for other bacteria which cause disease in humans.

Gram negative bacterial pathogens have a particular type of pathogenic mediator in the form of LPS. Whilst the lipid A part of this molecule is responsible for many of the pathological effects of infection such as inflammation, fever and intravascular coagulation, the repeating oligosaccharide units of the outer region (O-antigens) confer to the bacteria resistance against host defences such as the effects of complement and PMN bactericidal strategies. Alterations in the (LPS) structure of *Salmonella typhimurium* have been correlated with loss of virulence and changes in the interaction of the bacteria with macrophages and complement (Liang-Takasaki *et al* 1982, 1983). In this study, LPS preparations of all three of the virulent and avirulent strains of *L.pneumophila* were compared by SDS-PAGE and no differences were seen in the O-side chain ladder banding pattern. A biochemical alteration which would not be apparent by SDS-PAGE may have occurred in the LPS of the avirulent forms, but in previous studies (Conlan, 1987) has shown that there was no difference in the quantity or chemical composition of LPS preparations of CV and CAC. That study and the results reported here suggest that LPS is not a critical virulence determinant for *L.pneumophila* although it is probably involved in the disease process once infection has been established. The reaction of serogroup 1 *L.pneumophila* strains with certain monoclonal antibodies has been reported to be related to virulence.

Dournon *et al.*, (1986) found that the majority of environmental isolates reacted only with a monoclonal antibody designated type 1 while clinical strains reacted with type 2. The virulent and avirulent strains in this study both reacted with type 2 indicating that this reaction is not an *in vivo* virulence marker. This monoclonal antibody is directed at an LPS epitope so the identical reaction of virulent and avirulent forms also suggests that the virulence attenuation on culture medium does not affect LPS structure. The reaction of strains with MAb 2 indicates the likelihood of an isolate being environmental or clinical but it is not necessarily an indicator of the virulence of that strain. The avirulent bacteria used in this study were derived from clinical isolates and, whilst they had lost the appropriate mechanism to cause disease in guinea pigs, they had retained the epitope specific for MAb 2. It is possible that *Legionella* LPS undergoes minor changes during passage through the host which could account for an alteration of an epitope from that seen in the environment. In that case monoclonal antibody 2 is not a marker of the potential virulence of a strain but more likely provides evidence of strains which have caused disease.

Bacterial enzymes can also determine the severity of disease but some are also virulence factors. Strains of *Pseudomonas aeruginosa* which do not produce certain extracellular enzymes were less virulent in experimental models (Holder & Haidaris, 1979). When the extracellular enzyme activities of the *L.pneumophila* virulent and avirulent strains, grown *in vitro*, were compared, the avirulent strains had the same activities as the virulent strains. However, since the avirulent strains did not multiply *in vivo*, any pathogenic effects which the enzymes exert would not be exhibited. Thus the ability to produce a potential pathogenic factor *in vitro* is only relevant if that factor is produced in sufficient quantities *in vivo*. The possible role of extracellular proteases in the pathogenesis of LD is discussed in Chapter 4 of this thesis.

The role of flagella and pili in the disease process is very clear for pathogens which require these structures for attachment to host cells. The ability of enterotoxigenic *Escherichia coli* to adhere to epithelial surfaces in the intestine is mediated by pili which are therefore essential for establishment of infection. *Salmonella* strains depend on flagella for attachment and colonisation of the gut epithelial cells. A striking physical difference between virulent and avirulent strains in the present study was the 47kDa protein band seen following SDS-PAGE of the surface proteins. Further investigation demonstrated this to be flagellin and EM studies confirmed that virulent strains had far more flagella than their avirulent counterparts. Since *L.pneumophila* is a water born organism the possession of flagella would be more of an advantage for motility in that environment rather than to multiplication in lungs. The loss of flagella following culture on MHIH has been reported by Elliot and Johnson (1982) who concluded that flagella were not required for virulence in guinea pigs infected peritoneally. It would seem unlikely that flagella were important for virulence in the aerosol model since the shearing forces involved during aerosolisation of bacterial suspensions would probably have removed most, if not all, the flagella. Uptake of legionellae by macrophages was not dependent on flagella since the avirulent strains, with less flagella, were phagocytosed in similar numbers to virulent strains. Thus there does not appear to be a role for flagella in the early stages of disease but, following multiplication in the macrophage, the virulent organisms were very active and could be seen to move independently within the cells in a manner similar to that described by Rowbotham (1980) within amoebae. Once released from the macrophages these organisms were extremely motile (when observed by phase contrast microscopy) compared to the BCYE grown equivalent. The expression of large quantities of flagella during infection was confirmed by immunocytochemical staining of lung sections

with absorbed (to remove anti-LPS antibodies) anti-flagellin antiserum which revealed intense staining in the areas of infection. Increased motility in the lungs could be advantageous in allowing, for example, the bacteria to respond rapidly to chemotactic gradients which may aid survival and multiplication of *L.pneumophila*, *in vivo*.

If possession of flagella is not related to virulence the differences between the virulent and avirulent cells could be attributed to growth conditions and metabolic state of the organisms in the various environments examined. Several factors such as temperature, growth phase and nutrient availability are known to alter expression of flagella by *L.pneumophila* (Dennis, 1986, Mauchline, 1992) MHIH is a poor growth medium for *L.pneumophila* and those bacteria which do grow have reduced expression of flagella. Similarly, the legionellae seen moving rapidly within and out of macrophages in cell culture were more motile than the same organism grown on BCYE because the former were likely to be in a more active growth phase.

Another type of surface structure seen in these studies were fimbriae which were present on both virulent and avirulent strains indicating that attachment to surfaces is not important for *L.pneumophila* to cause disease.

Outer cell wall proteins are important for the structural integrity of bacteria, to facilitate passage of materials in and out of the cell via transmembrane channels and some are receptors or ligands. Membrane proteins of various intracellular pathogens have been identified as possible or actual virulence determinants. An outer membrane protein of *Salmonella typhimurium* responsible for the resistance of the organism to the oxygen-dependent killing mechanisms of polymorphonuclear neutrophils is an important virulence determinant for the organism (Stinavage *et al.*, 1990). The cross linking of outer membrane proteins of Chlamydia is important in determining the structure of the cell wall and the conversion between

infectious and non-infectious forms of the organism (Newhall and Jones, 1983, Bavoil *et al.*, 1984).

Several proteins in the outer cell wall of legionellae have been described (Ehret and Ruckdeschel, 1983, Gabay *et al.*, 1985, Pau *et al.*, 1988, Sampson *et al.*, 1990) and some have been suggested as pathogenic factors (Cianciotto *et al.*, 1989a). Two such proteins are the major outer membrane protein (MOMP) described by several workers (Ehret and Ruckdeschel, 1983, Hindahl and Iglewski, 1984, Butler *et al.*, 1985) and the macrophage infectivity potentiator (mip) protein identified by Cianciotto *et al.*, (1989b).

The role of the MOMP of *L.pneumophila* in virulence has been implied by its ability to bind to the complement receptor of macrophages which would facilitate uptake of the bacterium, a process essential for multiplication of legionella (Bellinger-Kawahara & Horwitz, 1990). In the present study there were no differences in phagocytosis of the virulent and avirulent strains by alveolar macrophages. Virulent and avirulent strains both expressed an outer cell wall protein with the characteristics of the MOMP i.e. a 29kDa band which was only observed on gels run under reducing conditions indicating a protein having interchain disulphide bonds. The MOMP was therefore not a virulence determinant of these strains and, whilst phagocytosis of *L.pneumophila* is required for virulence, it is not the only important factor since uptake of avirulent strains does occur.

The principal role of alveolar macrophages is to ingest and destroy foreign particles and invading micro-organisms therefore legionellae will tend to be phagocytosed by such cells. Intracellular survival and initiation of multiplication, on the other hand, require the bacteria to elaborate factors which overcome the natural destructive role of macrophages. The *Legionella* mip protein has been shown to have a key role in the initial stages of intracellular events by interfering with the oxidative response of macrophages by inhibiting phosphorylation of protein kinase C (Engleberg,

1993). However, mutants which do not express mip are only inhibited in the early stages of infection and intracellular multiplication of mutants occurs in a variety of cell types albeit more slowly than that of strains expressing mip (Cianciotto *et al.*, 1989b, Engleberg & Eisenstein 1991).

The avirulent strains in this study lost expression of a protein the same molecular weight as mip (24kDa) but the bacteria were defective in intracellular replication not just initiation of infection. Western blots of the avirulent strains stained with anti-mip antibodies confirmed that mip was expressed in avirulent strains. The loss of the 24kDa band in the avirulent strains was not a consistent finding either in repeat gels of the same outer membrane preparations nor when fresh batches were prepared. It is not clear why this is so, though several possibilities exist. A contaminating protein of similar molecular weight may have been present in avirulent strain preparations, the source of which could even have been a population of virulent organisms in the original suspension of bacteria from which the membranes were prepared. Mixtures of mutant and wild-type bacteria could be possible since the avirulent strains were derived from a single passage on MHIH and not by successive cloning of avirulent bacteria. Membranes prepared from suspensions of cloned bacteria might resolve this problem. Multiplicity of bands in this region of the gels resulted in uncertainty as to whether, in the later gels, the 24kDa band disappeared from virulent or appeared in avirulent strains. Proteolysis of the band occurring in only avirulent preparations seems unlikely. Variations in pouring consistent gradient gels could also have contributed to the problem because of poor resolution of bands. With the exception of mip there have been no reports of a virulent-strain specific 24 kDa band which may explain the irregularity of its appearance in this study.

To improve the reproducibility of the SDS-PAGE, pre-cast gradient gels for use with horizontal electrophoresis were used to analyse whole cells and

detergent extractions. The similarity of the protein profiles following a number of detergent extractions (figs 2.19 & 2.20) indicated that proteins on the surface of the bacteria were well conserved and varied little between virulent and avirulent forms. Horwitz (1987) did not find any differences in protein profiles between virulent and avirulent strains. The reaction of Western blots of the pairs of strains with various specific antisera also displayed a similarity in surface proteins. Antibodies in the sera against LPS may have confused these results since LPS can co-migrate with proteins as protein-LPS complexes during SDS-PAGE and give the appearance of a reactive protein band (Poxton *et al.*, 1985). This is demonstrated in fig 2.15 where Western blots of CV and CA reacted with MAb 2 in such a way as to suggest protein bands, yet the specificity of the MAb is for LPS.

The low molecular weight band found in Corby virulent strain, whole organisms and detergent-extracted pellets has not been described elsewhere but, since Stafford and Dodge avirulent strains and CAC contain the same band (Fig.2.20), it is unlikely that it is a virulence determinant.

The fact that the macrophages in the *in vitro* assay appeared undamaged by the avirulent strains, even when viable bacteria were present, suggests that the mere presence of bacteria does not harm the macrophage and growth of *L.pneumophila* is required for the cell destruction, possibly mediated by *Legionella* protease which has been detected intracellularly in guinea pig and human alveolar macrophages (Chapter 4, this thesis, Rechnitzer *et al.*, 1992). This again demonstrates that the possession of a single virulence or pathogenic factor by a micro-organism is not necessarily the key to causing its disease since a full complement of such factors is often required before infection is established.

The use of the macrophage assay enabled a characterisation of virulence but clearly other mechanisms are involved in the host response *in vivo*. The

next Chapter describes experiments which study the response of the guinea pig host to the virulent and avirulent strains.

CHAPTER 3

Virulence of *L.pneumophila* *in vivo*

3.1. INTRODUCTION

Removal of any one component that contributes to a pathogens' ability to cause disease may or may not result in an avirulent strain. The importance of such a factor depends as much on the host and prevailing circumstances *in vivo* as it does on the other contributing factors of the organism.

The environment of artificial culture media is, for many pathogens, different to that which they encounter *in vivo* yet the majority of investigations carried out on these pathogens are performed on bacteria grown on such media.

It is now clear that artificial growth media can drastically alter strains of *L.pneumophila* from the original clinical or environmental isolate. Chapter 1 of this thesis describes the results of passage of the Corby strain of *L.pneumophila* on MHIH and BCYE agars and confirms previous reports that avirulent forms of the organism are produced. However, despite extensive analysis of these strains a factor or factors responsible for loss of virulence could not be found. A number of environmental factors such as temperature (Ogg *et al.*, 1958; Maurelli & Sansonetti, 1988), and nutrients (Robinson *et al.*, 1983), have been found to influence the expression of bacterial virulence determinants and such parameters have also been shown to affect the virulence of *L.pneumophila* (Edelstein *et al.*, 1987). Several pathogens are known to express a different set of proteins when exposed to their *in vivo* environment to those expressed following growth on culture media. *Salmonella typhimurium* synthesizes several novel proteins in the presence of epithelial cells (Finlay and Falkow, 1989b) and upon infection of macrophages

(Buchmeier and Heffron, 1990). *Vibrio cholerae* recovered from the gut of infected animals expressed fewer cell envelope proteins than media-grown bacteria and these proteins were not expressed during *in vitro* culture (Jonson *et al*, 1989). Stress induced proteins which are produced as a result of nutrient depletion or exposure to toxic substances are also found. Hydrogen peroxide-induced proteins are believed to play a part in the pathogenesis of *S.typhimurium* (Fields *et al.*, 1986) and, given the intra-cellular environment in which virulent legionellae thrive and the known toxicity of hydrogen peroxide to *L.pneumophila* (Hoffman *et al.*, 1983) these may be induced in *L.pneumophila*. It is thus possible that the *in vitro* experiments which compared the avirulent with the virulent forms of Corby did not identify a factor associated with virulence because that was only induced *in vivo*.

A single passage of a strain on artificial media would be sufficient to switch off the expression of stress-induced proteins so it is important to recover bacteria directly from the *in vivo* environment. Using the guinea pig model of LD already described, virulent *L.pneumophila* were obtained from infected lungs and alveolar macrophages and compared with bacteria grown on artificial media in terms of their ability to infect macrophages and expression of surface proteins.

There is much evidence that the principal component of host defences with which *L.pneumophila* interacts is the macrophage and several mechanisms by which these bacteria can evade the anti-microbial defences of this cell have been proposed. However, the vast majority of these studies have been performed on macrophages cultured *in vitro*. Experiments described in Chapter 2 have shown that the Corby strain variants behave differently to each other within guinea pig macrophages which were cultured *in vitro*. The fates of these organisms *in vivo*, following aerosol administration were studied in order to investigate the role of macrophages in the early stages of infection with virulent and avirulent legionellae.

Whilst the role of macrophages in allowing multiplication of virulent *L.pneumophila* and providing protection from host defences, is well established, the contribution to bacterial clearance made by polymorphonuclear leukocytes (PNMLs) is in debate. It has been suggested that *L.pneumophila* is only partially susceptible to PMNL killing *in vitro* (Horwitz & Silverstein, 1981) but that avirulent mutants are more susceptible than their virulent counterparts (by inducing a greater oxidative response) (Summersgill *et al.*, 1988). However, virulent and avirulent strains are equally susceptible to the toxic effects of the myeloperoxidase-H₂O₂-halide systems of PMNLs *in vitro* (Locksley *et al.*, 1982; Lochner *et al.*, 1983; Jepras & Fitzgeorge, 1986) and depletion of PMNLs in the guinea pig model of LD results in a decrease in LD₅₀, demonstrating that these cells are important in defence against *L.pneumophila in vivo* (Fitzgeorge *et al.*, 1988). In an attempt to clarify the role of these cells, the interaction of PMNLs with virulent and avirulent forms of the Corby strain following aerosol infection of guinea pigs was studied in parallel with macrophages. In conjunction with conventional cell separation and bacterial culture techniques the use of flow cytometry to monitor the cellular response of guinea pigs was assessed. Lung phagocytes were identified by their size and granularity and antibody markers used to fluorescently label cells and intracellular bacteria.

3.2. MATERIALS AND METHODS

3.2.1. ISOLATION OF *IN VIVO*-GROWN *L.PNEUMOPHILA*.

Virulent Corby (CV) and Stafford (SV) strains were incubated for 48 hours with macrophages in tissue culture, as described in Chapter 1. The culture supernatant was then removed and any bacteria which were present were collected by differential centrifugation (200g to pellet cell-debris then 1000g to pellet bacteria). The macrophages were repeatedly freeze-thawed to release intracellular organisms which were collected by centrifugation as above.

CV was also obtained from macerated lungs of guinea pigs with experimental LD. Lungs were homogenised and bacteria pelleted from the supernatant following centrifugation to remove tissue debris. Bacteria were also isolated from homogenate supernatants using magnetic (Dynal) beads. The bacteria were coated with a rabbit anti *L.pneumophila* serogroup 1 LPS IgG then incubated with magnetic beads conjugated to anti-rabbit IgG antibody. The bacteria-coated beads were removed from homogenate supernatants by means of a strong magnet and, following washing in buffer, the bacteria were released from the beads by incubation at 37°C for 1-2 hours.

3.2.2. PREPARATION OF ANTISERA TO *IN VIVO* GROWN *L.PNEUMOPHILA*.

Guinea pigs were immunised with bacteria which had been isolated from lungs of guinea pigs infected with CV and SV. Each suspension, containing approximately 10^7 live bacteria/ml (by total count) was diluted in an equal volume of Freund's incomplete adjuvant and mixed to produce a smooth emulsion. Three guinea pigs were injected intra-muscularly (in the hind legs) with 200µl of the emulsion. After 2 weeks the animals were given the same

doses as above and blood was obtained a week later. Sera were tested by immunostaining of Western blots of BCYE grown and *in vivo* grown bacteria (see below).

3.2.3. ANALYSIS OF *IN VIVO* GROWN LEGIONELLAE

The uptake and intracellular replication of *in vivo* grown CV by guinea pig alveolar macrophages was compared with that of BCYE-grown CV using the intracellular growth assay described in Chapter 1.

SDS-PAGE analysis was performed in gradient 10-20% acrylamide gels which were then Western blotted as described in Chapter 1.

Nitrocellulose blots were immunostained with the following:

- a) antisera raised in rabbits against CV and SV grown on BCYE;
- b) convalescent sera from guinea pigs challenged with virulent and avirulent strains;
- c) antisera raised in guinea pigs against *in vivo* grown CV and SV.

3.2.4. CELLULAR RESPONSE OF GUINEA PIGS TO VIRULENT AND AVIRULENT CORBY.

Guinea pigs were challenged with aerosols of CV, CA and CAC as described in Chapter 1. At 0, 4, 8, 12, 24 and 30 hours following infection 3 guinea pigs were sacrificed, Lungs of two of the animals were washed out with tissue culture medium (MEMa, see Chapter 1). The lungs were removed from the third and fixed in phosphate buffered formalin for histopathology.

The volume of each lavage was measured then aliquots removed for bacterial viable counts. Counts were performed on the whole lavage and, by separation of cells and supernatant, on the intra- and extra-cellular environments. The remainder of the lavage was centrifuged to pellet the cells which were resuspended in 0.5 ml of MEMb (see Chapter 1). Small

volumes were removed for total white cell counts and cell smears which were stained with Leishmans differential white cell stain. A portion of the cells was taken into each of several tubes for immunolabelling (see below) and the remainder diluted in 1 ml of MEMb and overlaid on a cell density-separation medium (J prep).

3.2.5. IMMUNOLABELLING OF CELLS FOR FLOW CYTOMETRY

Monoclonal antibodies with specificities for the guinea pig cells under study were not available, so the following were tested for cross-reaction: mouse monoclonal anti-human HLA-DR, FITC conjugate (Dako) and mouse monoclonal anti-human granulocyte, FITC conjugate (Dako). A mouse monoclonal antibody of the same isotype, FITC conjugate negative control (Dako) was used with these antibodies. To label bacteria, a rabbit anti-*L.pneumophila* serogroup 1 LPS, FITC conjugate was used with rabbit IgG, FITC conjugate negative control. Cells were incubated with antibodies at room temperature for 30 minutes after which time, 3 ml of PBS was added and the cells pelleted to wash off excess antibody. The pellets were resuspended in 0.5 ml PBS containing 1% formaldehyde to fix the cells and kill any bacteria.

3.2.6. FLOW CYTOMETRY

Samples were analysed in collaboration with Dr L.A.E. Ashworth with the skilled assistance of Mrs.S.A. Sharpe on a Coulter EPICS PROFILE

3.3. RESULTS

3.3.1. ISOLATION OF *IN VIVO*-GROWN *L.PNEUMOPHILA*

Isolation of *L.pneumophila* from macrophages in tissue culture was more successful than removal from infected guinea pig lung since macerated lung material was difficult to remove by centrifugation. The use of magnetic (Dynal) beads improved the separation but, as this method involves binding antibodies to the bacterial surface, these bacteria could not be used for Western blotting. The quantities of *in vivo*-grown bacteria were subsequently very low and there was only sufficient of the Stafford strain to immunise guinea pigs. The remaining experiments were performed using CV isolated from macrophages in tissue culture.

3.3.2 UPTAKE AND INTRACELLULAR GROWTH OF *IN VIVO* GROWN CV.

The uptake of *in vivo*-grown CV by guinea pig alveolar macrophages in tissue culture was similar to that of the same strain grown on BCYE. Fig. 3.1 shows that for both types of bacteria the majority of the bacteria were ingested after 20 minutes incubation and that further uptake occurred at similar rates.

Intracellular multiplication of the media-grown CV occurred at a faster rate than the *in vivo*-grown CV (Fig.3.2.) but, given the variations in replication rate of strains in this assay (see Fig 2.3., Chapter 2), the difference may not be significant.

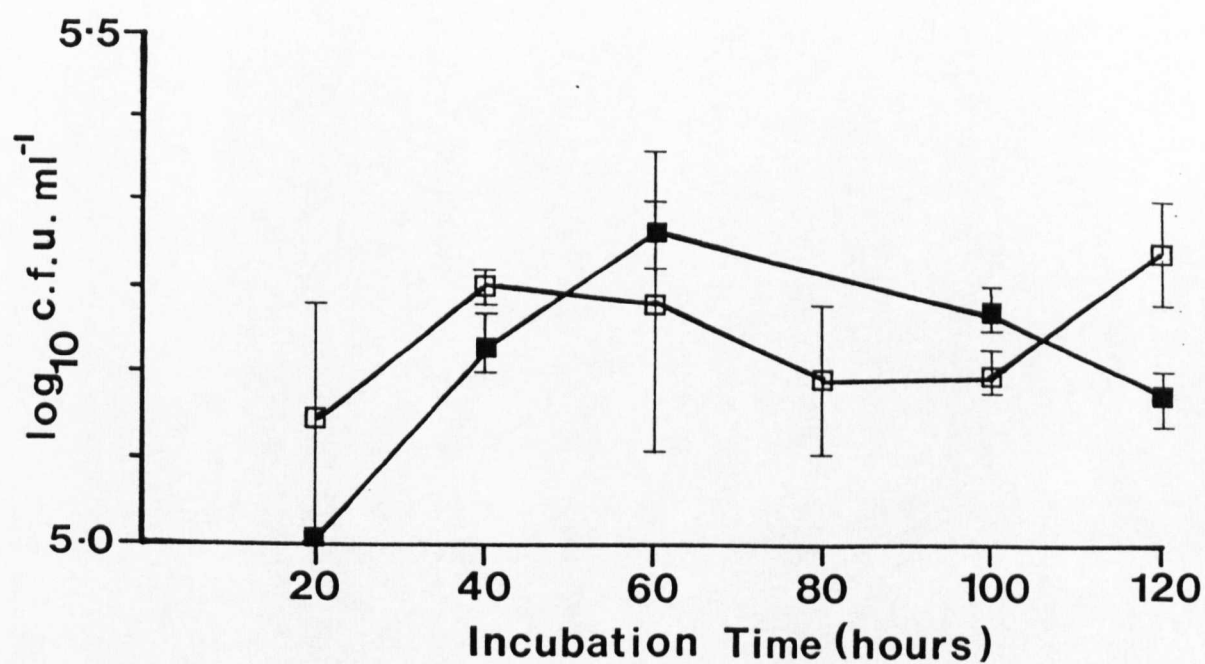


Fig.3.1 Viable counts of *in vivo*-grown and BCYE-grown Corby associated with guinea pig alveolar macrophages in tissue culture during 120 minutes incubation.

□- CV *in vivo*; ■- CV BCYE.

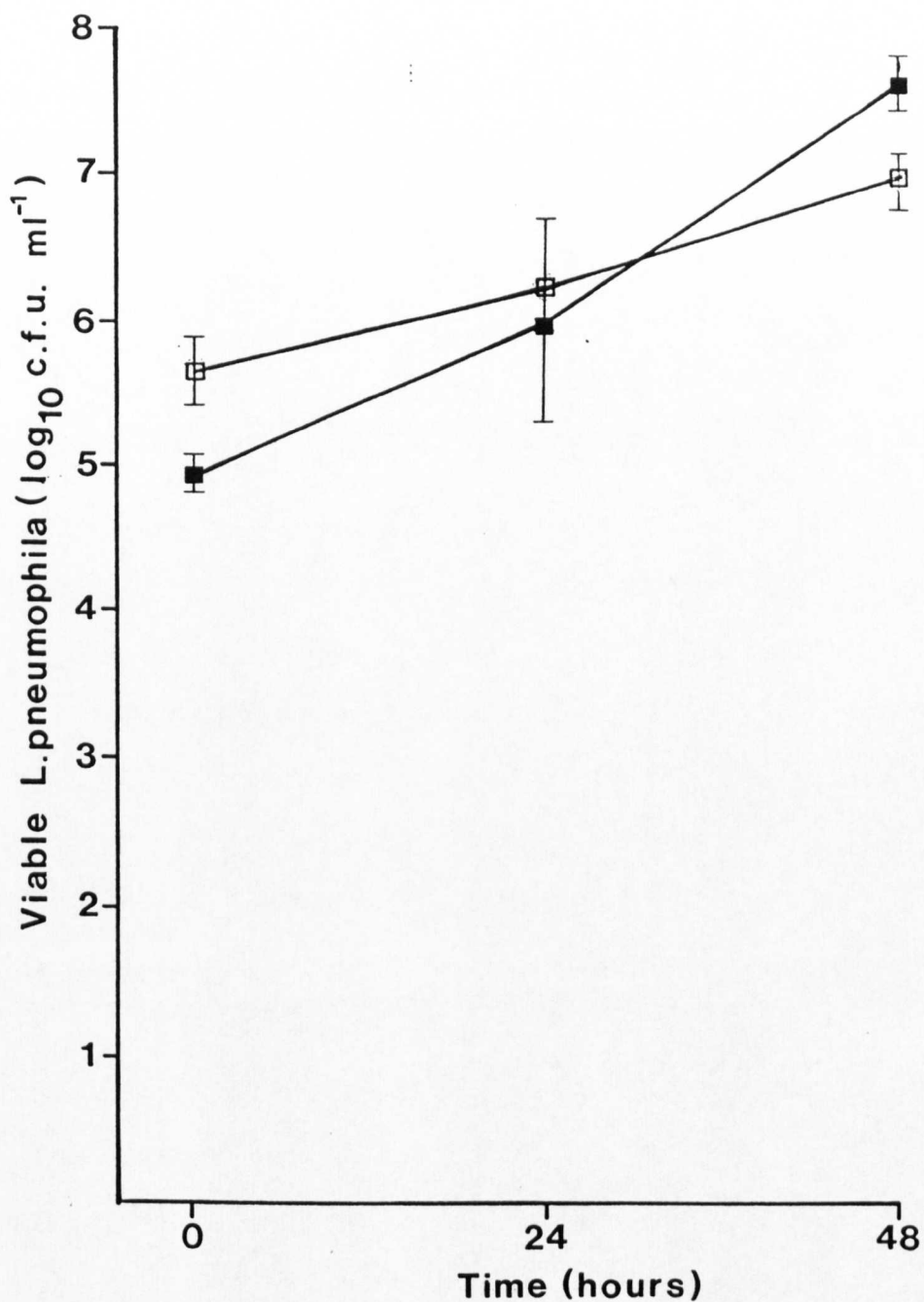


Fig.3.2 Viable counts of *in vivo*-grown and BCYE-grown Corby (CV) multiplying within guinea pig alveolar macrophages in tissue culture incubated for 0, 24 and 48 hours.

□ - CV *in vivo*; ■ - CV BCYE.

3.3.3. SDS-PAGE AND WESTERN BLOT ANALYSIS OF CV, GROWN *IN VIVO*

Insufficient quantities of *in vivo* grown SV were obtained for separation by SDS-PAGE but antiserum was raised against these bacteria. The results of immunolabelling blots of BCYE-grown bacteria with various antisera are shown in Fig.3.3. Fewer antigens were recognised by antiserum against *in vivo*-grown SV (Lanes 9 and 10) than by that raised in rabbits against BCYE-grown SV strains (lanes 1-4) and even fewer by antiserum which had been raised against SA recovered from macrophages. Similarly, the sera taken from guinea pigs infected with the virulent and avirulent strains (lanes 5-8) reacted with fewer bands than the rabbit sera but showed some differences to the pattern of staining with antisera to *in vivo* grown bacteria.

A low molecular weight band was observed on both virulent and avirulent blots which stained only when incubated with anti-*in vivo*-grown SV.

Contaminating proteins from the tissue culture medium prevented interpretation of SDS-PAGE gels of *in-vivo*-grown CV stained for total protein, therefore analysis was restricted to immunolabelling of Western blots. Fig.3.4 is a comparison of blots of BCYE-grown CV (odd-numbered lanes) with *in vivo*-grown CV (even-numbered lanes) stained with the same types of antisera described above. In general, fewer protein bands were found on *in vivo* CV blots than the paired BCYE CV blots and no bands unique to *in vivo*-grown CV were apparent.

In vivo-grown CV appeared to demonstrate a reduced antigen expression compared with BCYE-grown CV. Antibodies raised against *in vivo* CV reacted strongly with bands at around 19kDa and 16kDa and also in the region of the 36 and 45 kDa markers and it was only in these areas that the *in vivo* grown blots reacted

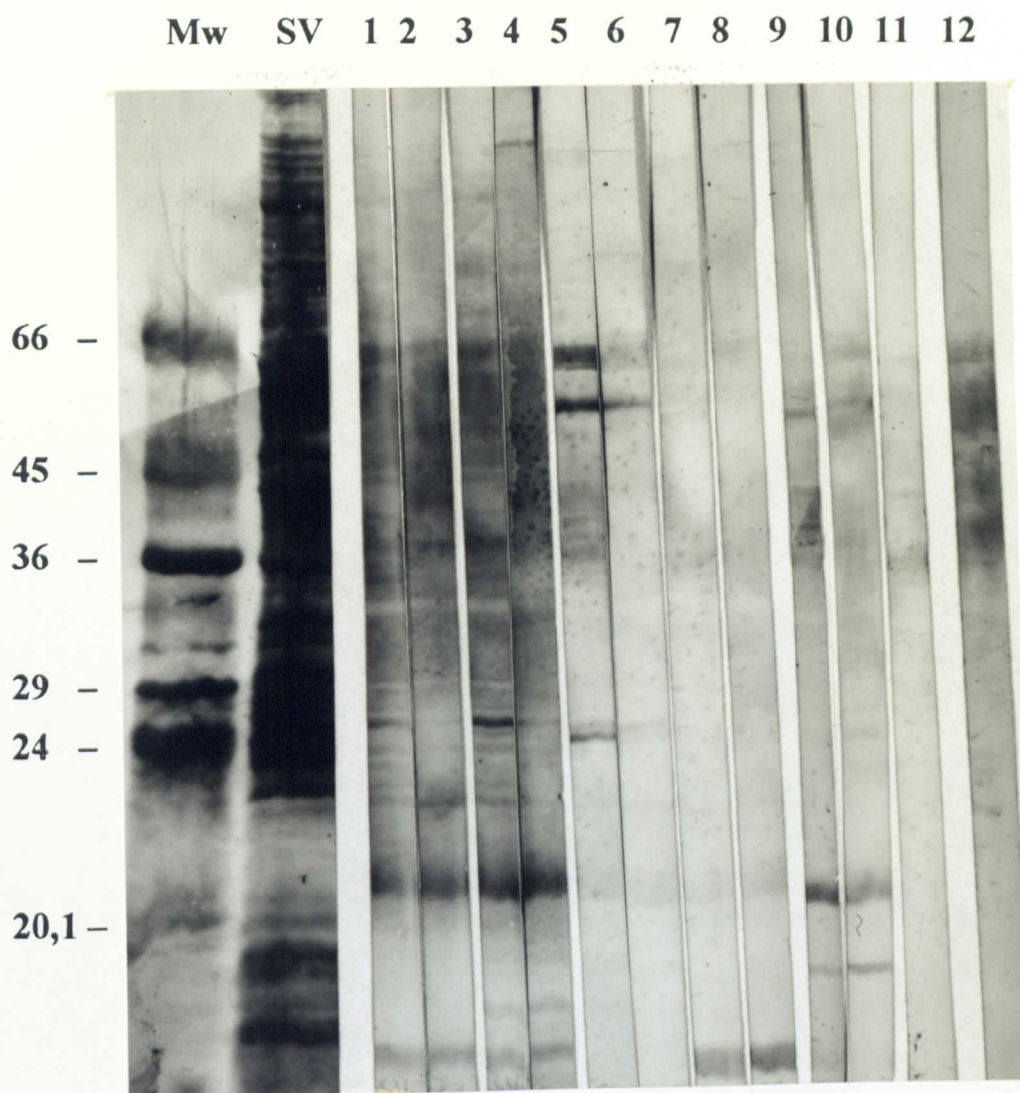


Fig.3.3 Western blot of Stafford virulent (SV, odd numbered lanes) and avirulent (SA, even numbered lanes) strains immunostained with the following antisera:

- 1 & 2, rabbit anti-SV whole bacteria;
 - 3 & 4, rabbit anti-SA whole bacteria;
 - 5 & 6, serum from guinea pigs infected with SV;
 - 7 & 8, serum from guinea pigs infected with SA;
 - 9 & 10, guinea pig anti-*in vivo* grown SV;
 - 11 & 12, guinea pig anti-SA recovered from infected guinea pigs.
- Molecular weight markers and SV incubated with colloidal gold total protein stain.

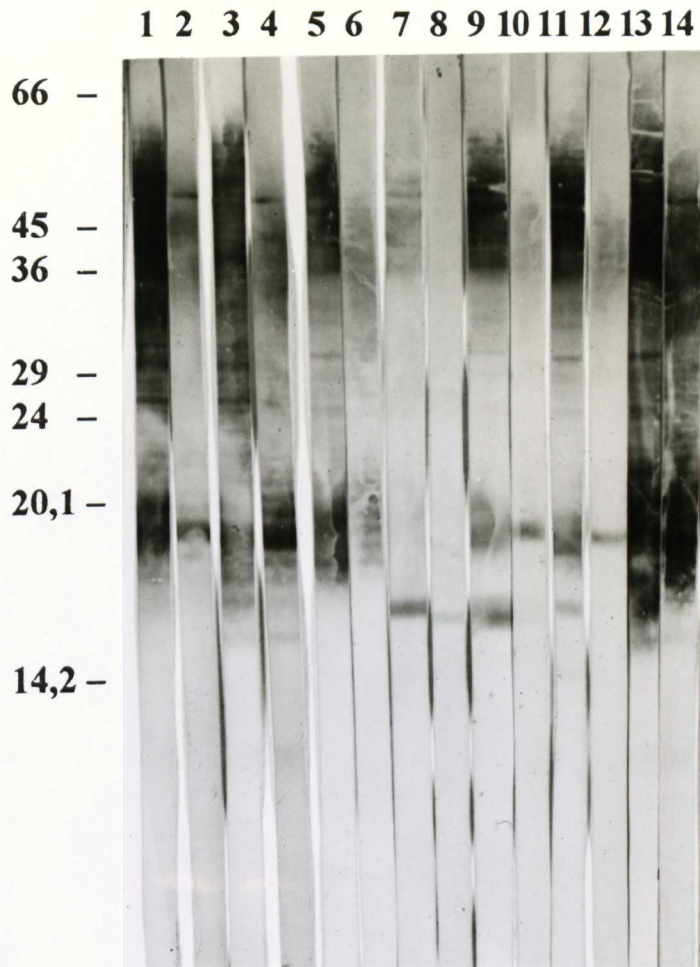


Fig.3.4. Western blot of BCYE-grown Corby (CV, odd numbered lanes) and *in vivo*-grown Corby (even numbered lanes) immunostained with the following antisera:

- 1 & 2, rabbit anti-CV whole bacteria;
- 3 & 4, rabbit anti-CA whole bacteria;
- 5 & 6, guinea pig anti CA recovered from infected guinea pigs;
- 7 & 8, serum from guinea pigs infected with CV;
- 9 & 10, serum from guinea pigs infected with CA;
- 11 & 12, guinea pig anti-*in vivo* grown Corby;
- 13 & 14, rabbit anti-*L.pneumophila* serogroup 1 LPS.

3.3.4. CELLULAR RESPONSE OF GUINEA PIGS TO VIRULENT AND AVIRULENT CORBY

Over a period of 3 months small volumes of guinea pig lung lavages (which were performed for various experiments) were taken and cell counts performed. The results are given in Table 3.1. and show that, on average, the cell population consisted of around 60% macrophages and 40% granulocytes comprising PMNLs and eosinophils.

Table 3.1. Analysis of normal guinea pig lung lavages, total and differential white cell counts.

	Total cell count (/ lung)	Proportions of cell types (%)	
		Granulocytes	Macrophages
Mean	1.02×10^6	41.9	57.9
Range	$0.3-1.8 \times 10^6$	19-55	45-81
Number of animals	16	20	20

The changes in the relative proportions of these cell populations which occurred following aerosol challenge of the animals with CV, CA and CAC are shown in Fig.3.5. The PMNL population increased initially after challenge with all three strains but after 4 hours these cells returned to normal levels in animals given CA and CAC yet in animals given CV the proportion of PMNLs rose further and became the dominant cell type (Fig. 3.5a). The eosinophil content of the lavage varied little throughout the course of infection with all three strains (Fig.3.5b).

The proportion of macrophages showed an initial fall following infection with all three Corby strains and remained lower than normal in guinea pigs infected with CV but in animals given CA and CAC the

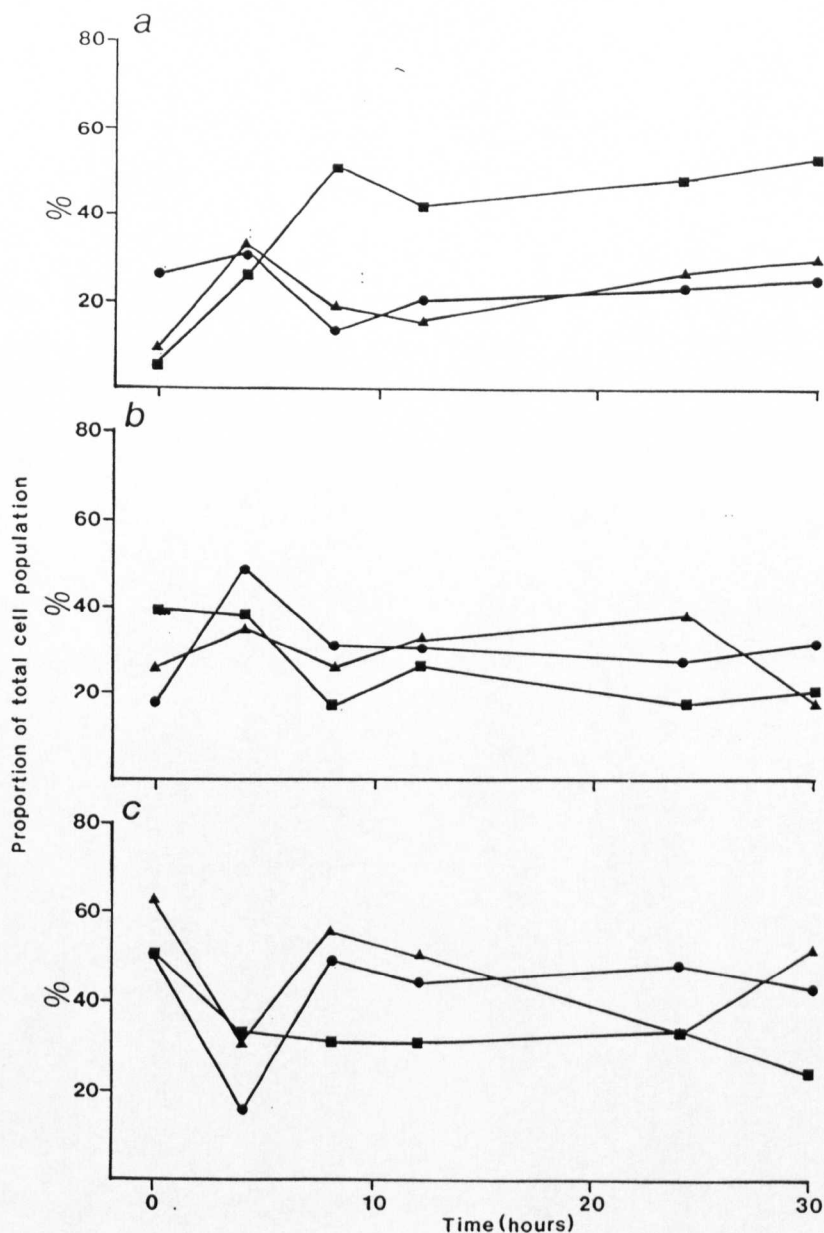


Fig.3.5. Changes in alveolar cell populations following infection with virulent and avirulent forms of Corby over 30 hours, based on differential white cell counts.

- a. PMNs as a percentage of total population;
- b. eosinophils as a percentage of total population;
- c. macrophages as a percentage of total population.

■ - Corby virulent (CV), ● - Corby avirulent, MHIH-derived (CA),
 ▲ - Corby avirulent, BCYE-derived (CAC).

percentage rose back to normal levels. Fig.3.6 is a graph of PMNLs as a percentage of the combined PMNL and macrophage populations and demonstrates the marked increase in these cells in the CV infected animals compared to little change in the CA and CAC infected animals (apart from an initial peak at 4 hours). Fig 3.7 demonstrates that the increase in the PMNL population resulted in an increase in the total numbers of cells in the lungs in CV infected animals.

3.3.5. INFECTIVITY OF VIRULENT AND AVIRULENT CORBY STRAINS *IN VIVO*

Bacterial, viable counts performed on whole, macerated lungs taken at each time interval from the three groups of guinea pigs are shown in Fig.3.8. Up to 12 hours post infection CV did not increase in numbers then rapid multiplication occurred such that at 30 hours counts were 100 fold greater than at 0 hours. Both CA and CAC counts declined gradually over 30 hours. Fig.3.9 is a graph of the bacterial numbers in whole lung lavage which were similar to those counted in whole lung macerates apart from some slight differences such as a decrease in CV up to 8 hours and a slight rise of CA from 0-8 hours. Numbers of intra- and extracellular bacteria were also calculated and are given in Fig.3.10. The bacteria in the CV-infected lungs remained mostly intracellular. CAC was predominantly intracellular up to around 18 hours then switched to being mainly extracellular. Apart from the first 4 hours following infection more viable CA were found within cells than extracellularly and the difference between the counts became greater with time.

The differences in viable counts of intra- and extra-cellular CA and CAC from 0 hours to each time point are shown in Fig 3.11. The predominance of intracellular CA is again clear from this graph.

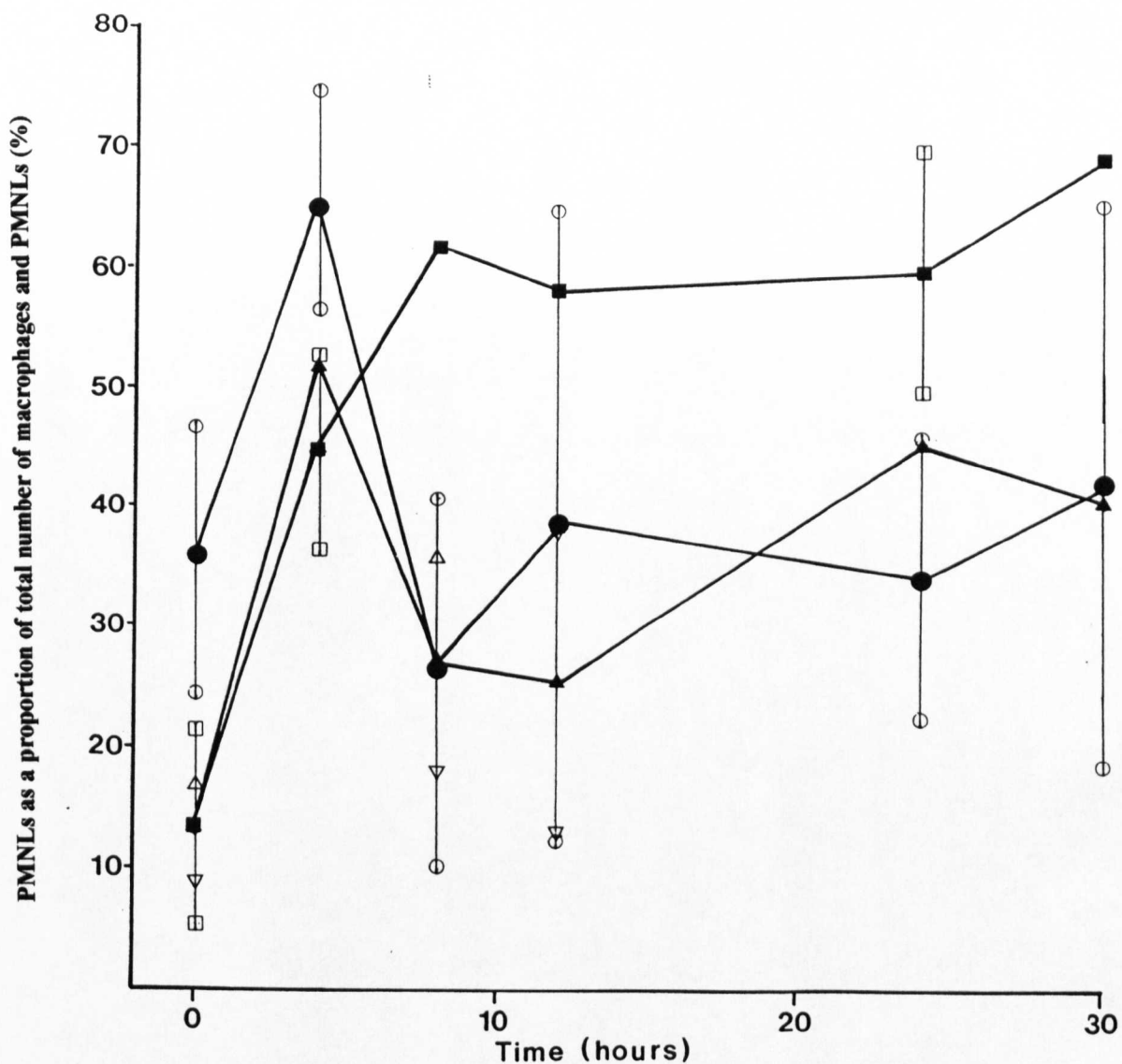


Fig.3.6. Changes in the relative proportions of PMNs calculated as a percentage of the combined PMN and macrophage population following infection with virulent and avirulent Corby over 30 hours. Each point represents the mean of two animals

■ - CV; ● - CA; ▲ - CAC.

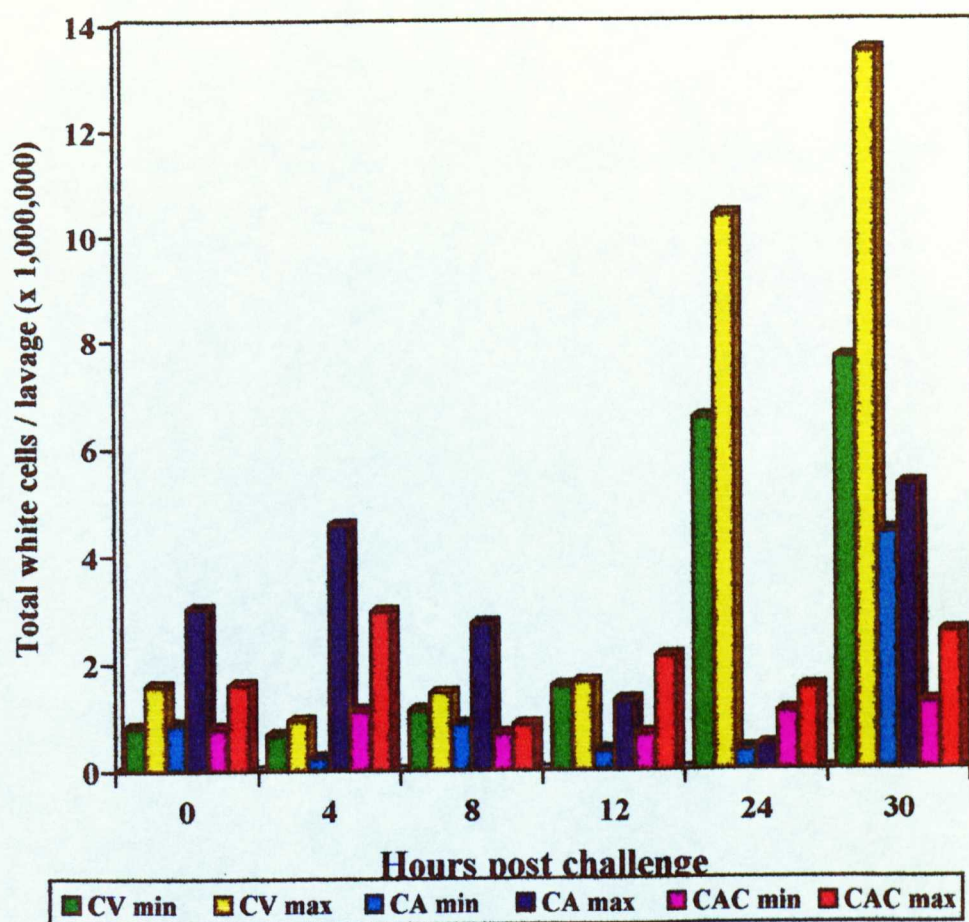


Fig.3.7 Total white cell counts on guinea pig lung lavages following aerosol challenge with virulent and avirulent Corby.

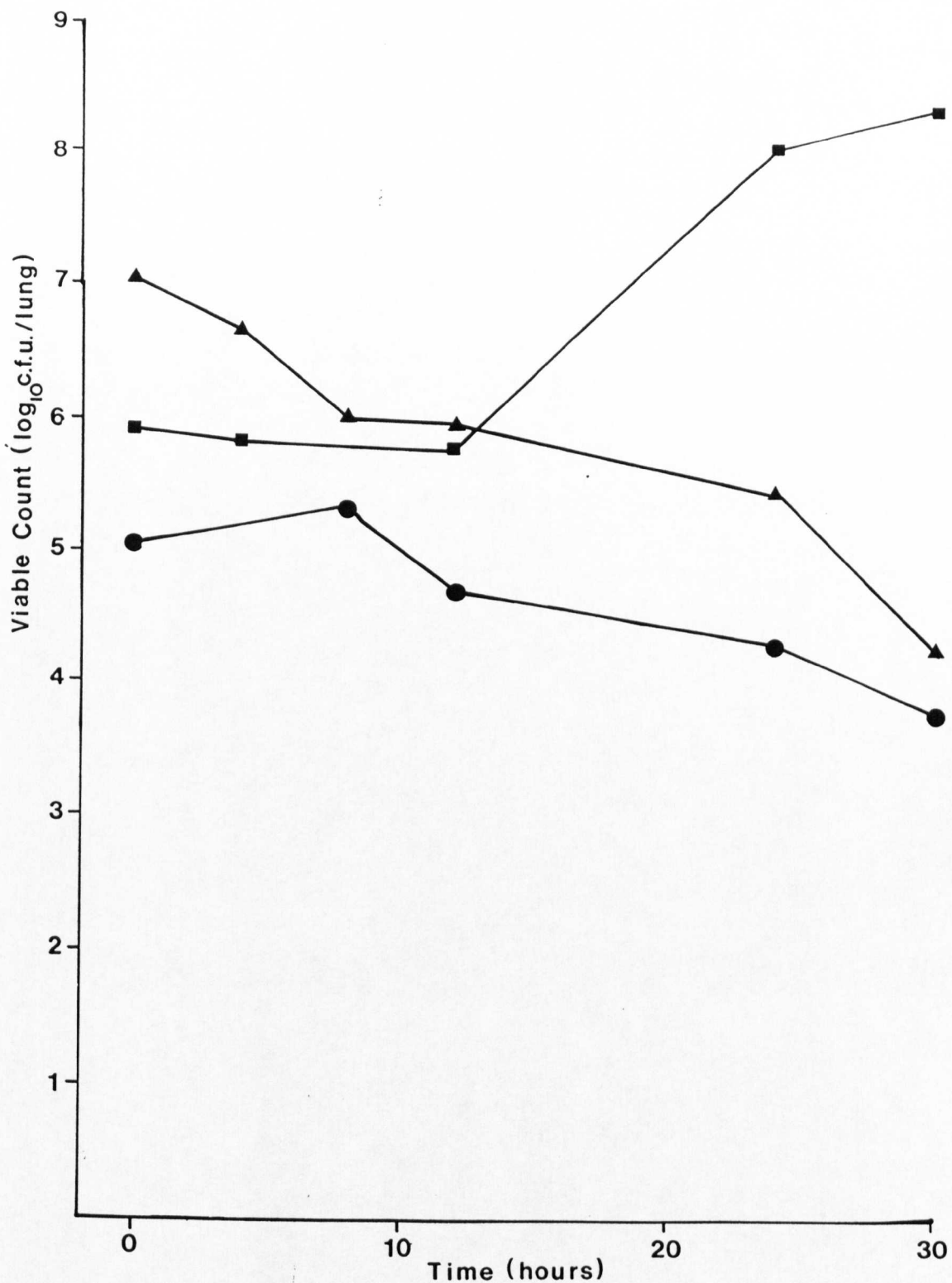


Fig.3.8. Viable bacterial counts of whole, macerated lungs of guinea pigs following infection with Corby strains. Each point represents one animal.

■ - CV; ● - CA; ▲ - CAC.

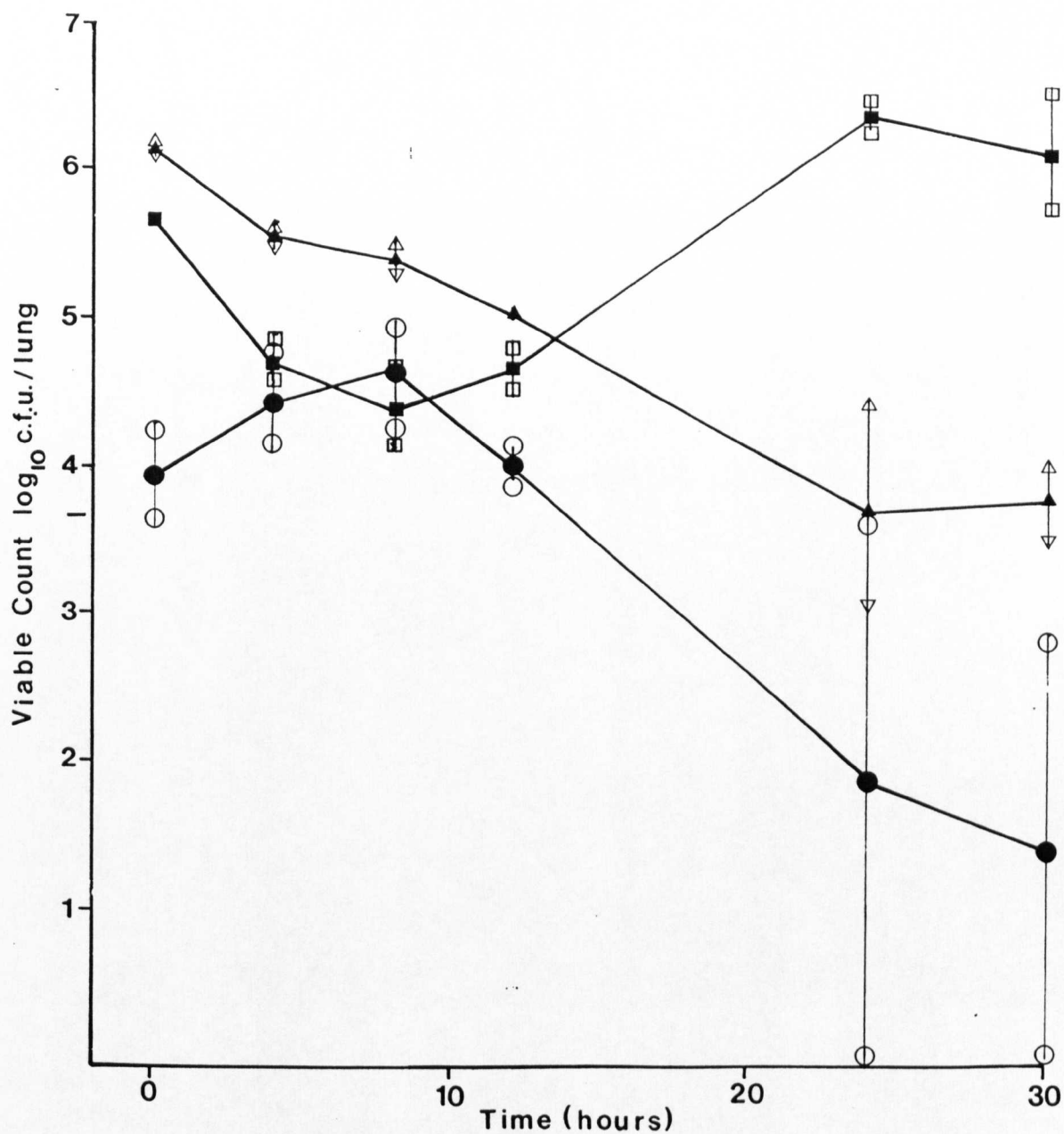


Fig.3.9. Viable bacterial counts of whole lung lavages from guinea pigs infected with Corby strains. Each point represents the mean of two animals. Open symbols represent range of values.

■ - CV; ● - CA; ▲ - CAC.

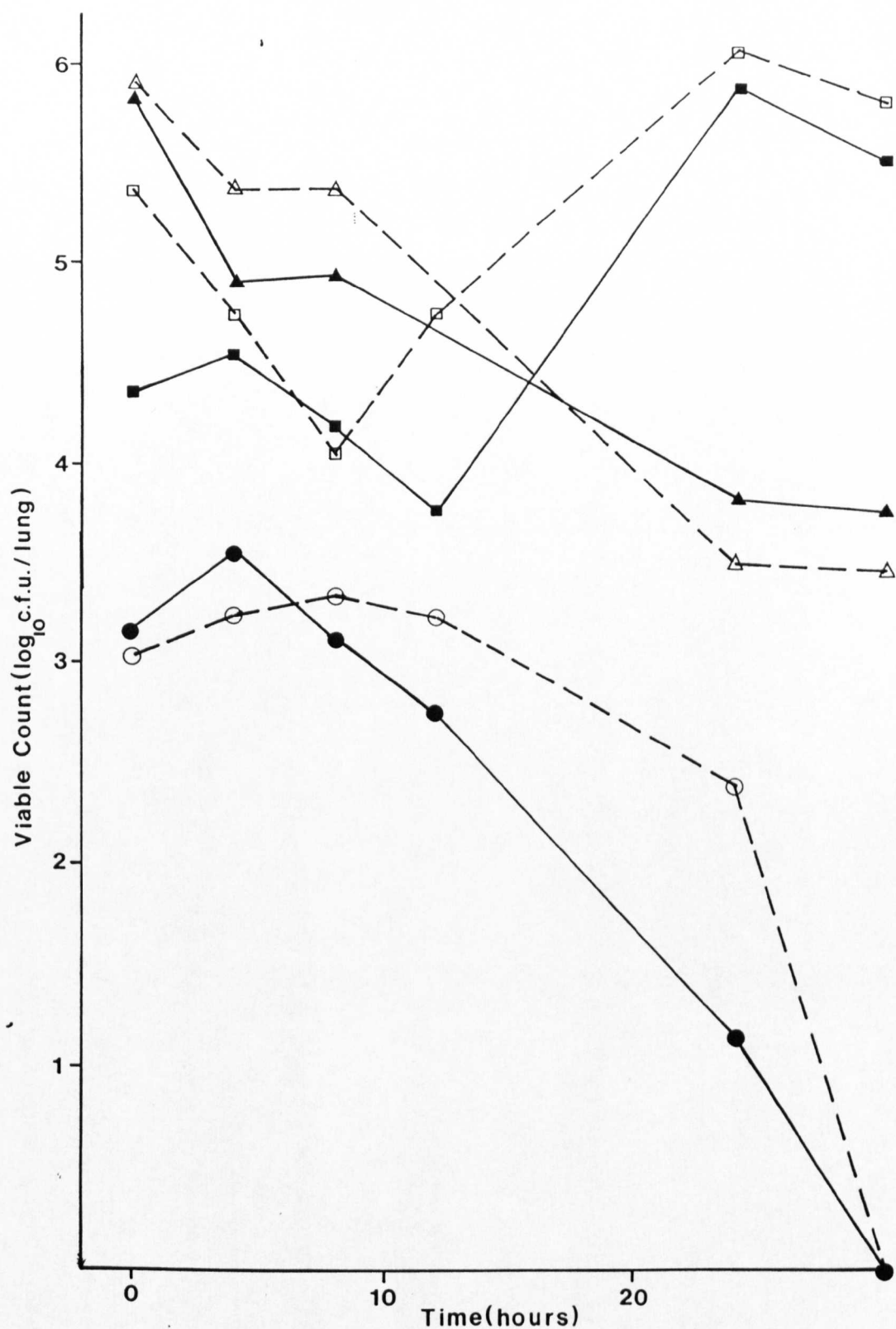


Fig.3.10. Viable bacterial counts of the cell associated (----) and extracellular (—) fractions of guinea pig lung lavages following infection with the Corby strains.

■ - CV; ● - CA; ▲ - CAC.

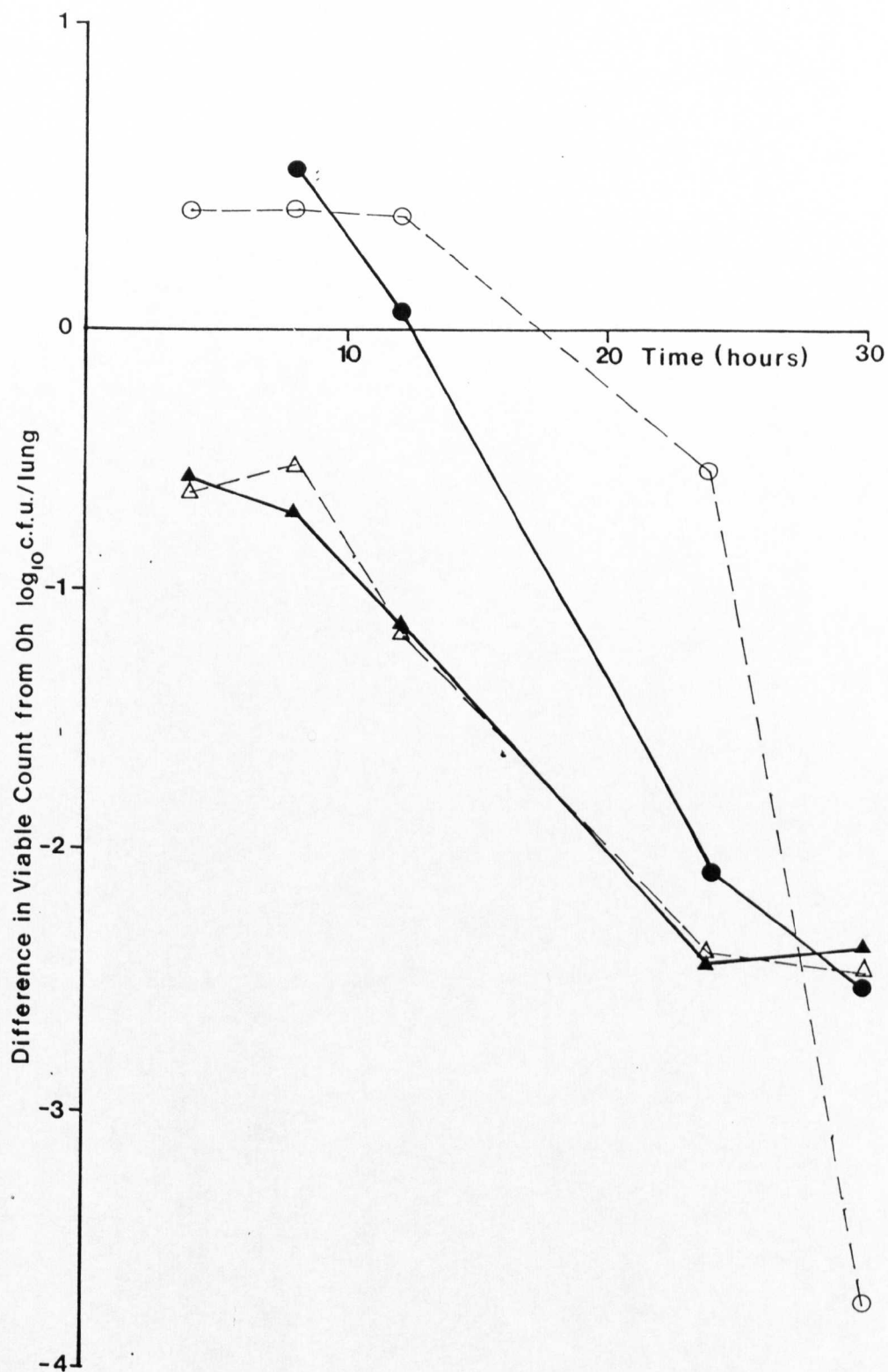


Fig.3.11. Difference in intra(----) and extracellular (—) viable bacterial counts of guinea pig lung lavages at each time point following infection with CA and CAC as compared with the counts at 0 hours.

● - CA;

▲ - CAC.

There was very little change in the relative numbers of intra and extracellular CAC from 0-30 hours but the rate at which the viability of this strain decreased was similar to CA and greater than was apparent from Fig.3.10.

3.3.6. HISTOLOGY

A subjective score of the numbers of eosinophils in the lungs following infection was made by veterinary histopathologist Dr. G. Hall and is shown in Table 3.2. This analysis was performed in order to check the results obtained by stained smear cell counts.

Table 3.2. Numbers of eosinophils in Haematoxylin and eosin stained sections of lungs of guinea pigs following aerosol infection of virulent and avirulent Corby.

Time (hours)	Subjective score of number	
	VIRULENT	AVIRULENT
0	+	+
4	+	+++
12	+++	++
24	++	+
30	++	+

3.3.7 FLOW CYTOMETRY

The results obtained by flow cytometry were difficult to analyse due to low numbers of cells in several samples (which prevented comparisons of successive and duplicate tests), the similarity of the macrophages and PMNLs in terms of size and granularity and the high non-specific binding of antibodies to these cells. Thus a complete study of the progression of changes in the cell populations was not possible but the histograms shown

in Figs 3.12-3.18 show that labelling of macrophages and PMNLs was possible in some samples.

Fig 3.12 is the data obtained when a 0 hour sample was incubated with a negative control antibody and anti-human HLA-DR antibody (for labelling macrophages). The top histograms show the total number of 'events' (i.e. cells and particulate material) recorded and distributed in terms of their size (forward scatter, FS) and granularity (side scatter, SS). The distribution was very diverse and it can be seen that a large proportion of the events were in area 1 which was found to be debris. Through trial and error the area encircled by the bit-map was found to be the most likely position to find events carrying the specific FITC label. This can be seen on the lower histograms which register the percentage of events within the bitmap which were labelled. The problem most often encountered with this technique is illustrated in Fig.3.13, that of inconsistency between samples from the two guinea pigs at each time point. In this case the macrophage population bit-map set at 0 hours located labelled cells in one sample but not the other. Fig 3.14 is the data from a 12 hour CV infected sample and shows a change in the distribution of events in the top histogram. The 'sausage'-shaped area contained a greater number of events and was later shown to be PMNLs (unfortunately the granulocyte label did not work at this stage). There was also a shift in the macrophage population which resulted in the movement of the bit-map in the direction of increased side-scatter which indicated an increase in the granularity of the cells. The data shown in Figs 3.15-3.17 are of CV-infected lung lavages from a separate experiment. In an attempt to simultaneously label the cell types the FS and SS scales were altered to try and differentiate the overlapping macrophage and PMNL populations. Fig 3.15 confirms that events in the 'sausage'-shaped area did carry the granulocyte antibody label. Fig 3.16 shows how

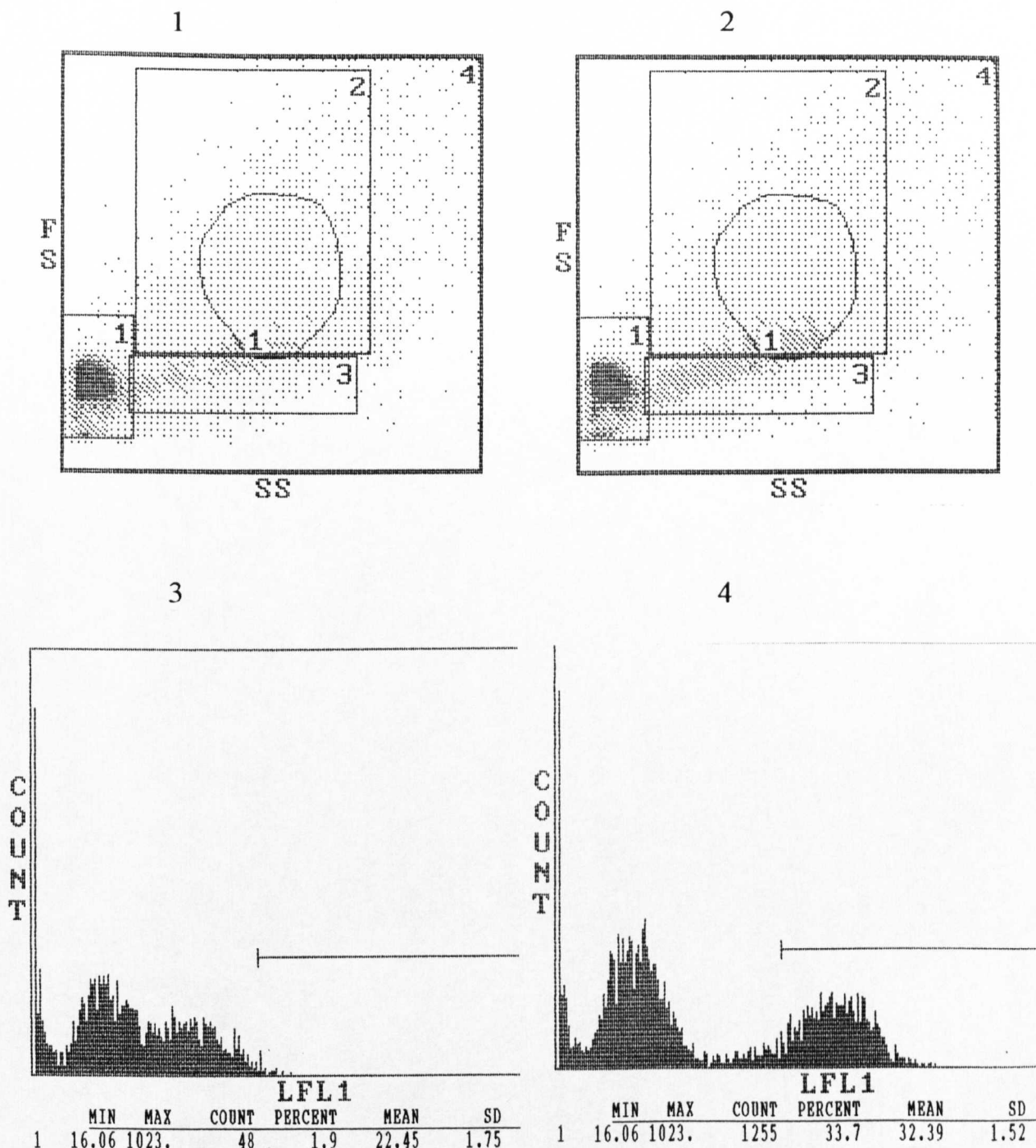


Fig.3.12. Flow cytometric analysis of guinea pig lung lavage immediately following infection with CV (0 hours). The histograms on the left are a negative control for the sample incubated with anti-HLA-DR antibody (FITC-linked) on the right. Histograms 1 and 2 show the distribution of particles in the sample in terms of size and granularity. Histograms 3 and 4 show the percentages of particles within the circular bit-map which fluoresce. Thus 33.7% of particles fluoresced following incubation with anti-HLA-DR compared with 1.9% in the control.

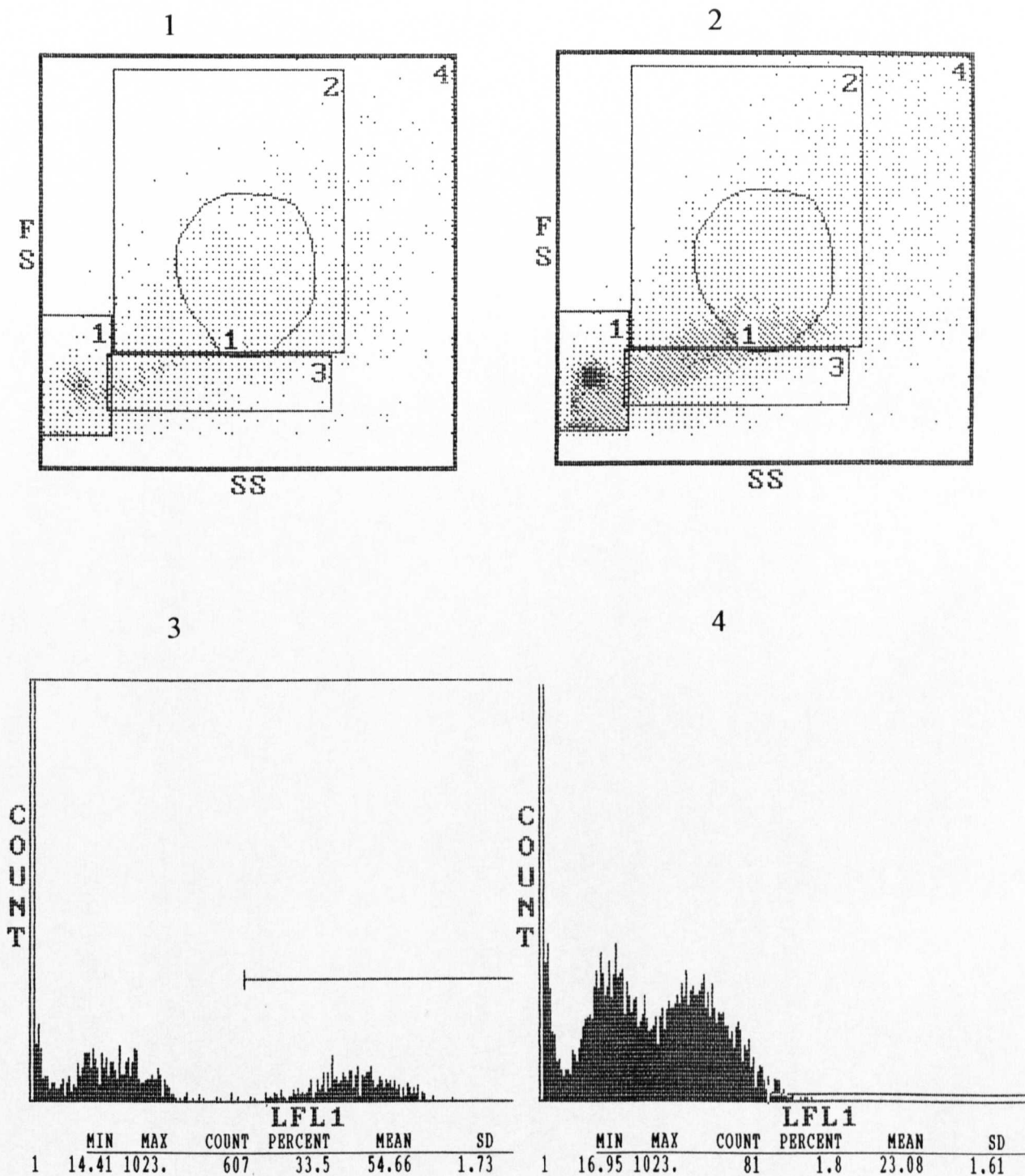


Fig.3.13. Flow cytometric analysis of guinea pig lung lavage taken 4 hours post-infection with CV. Histograms as for Fig.3.12 except both are samples from duplicate animals incubated with anti-HLA-DR showing the problem of differences in the cells between samples. The control sample gave a background fluorescence of 1.7%.

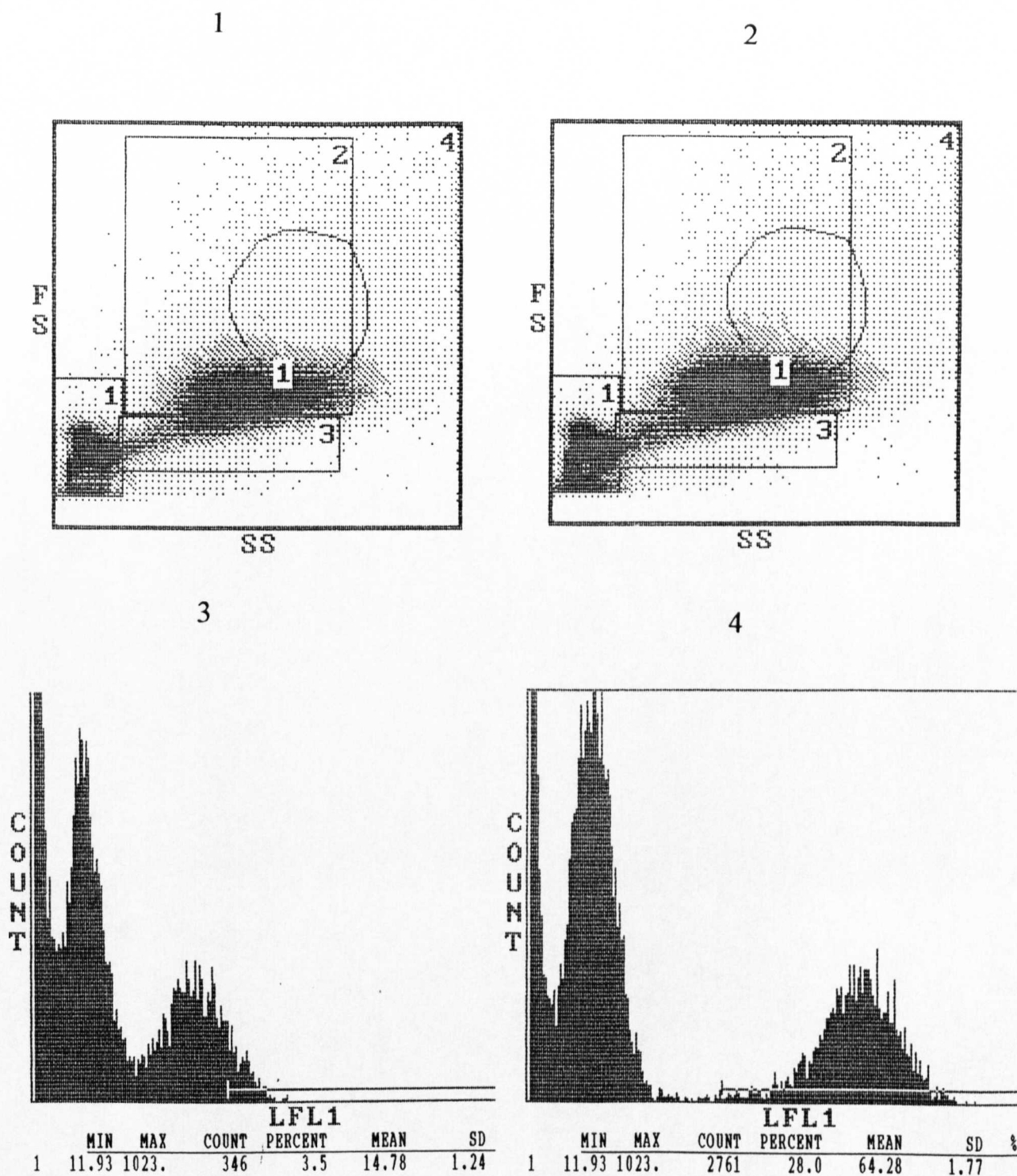


Fig.3.14. Flow cytometric analysis of lung lavage taken from a guinea pig 12 hours following infection with CV. Histograms as Fig.3.12 but with an altered bit-map to accommodate movement of the macrophage (HLA-DR positive) population.

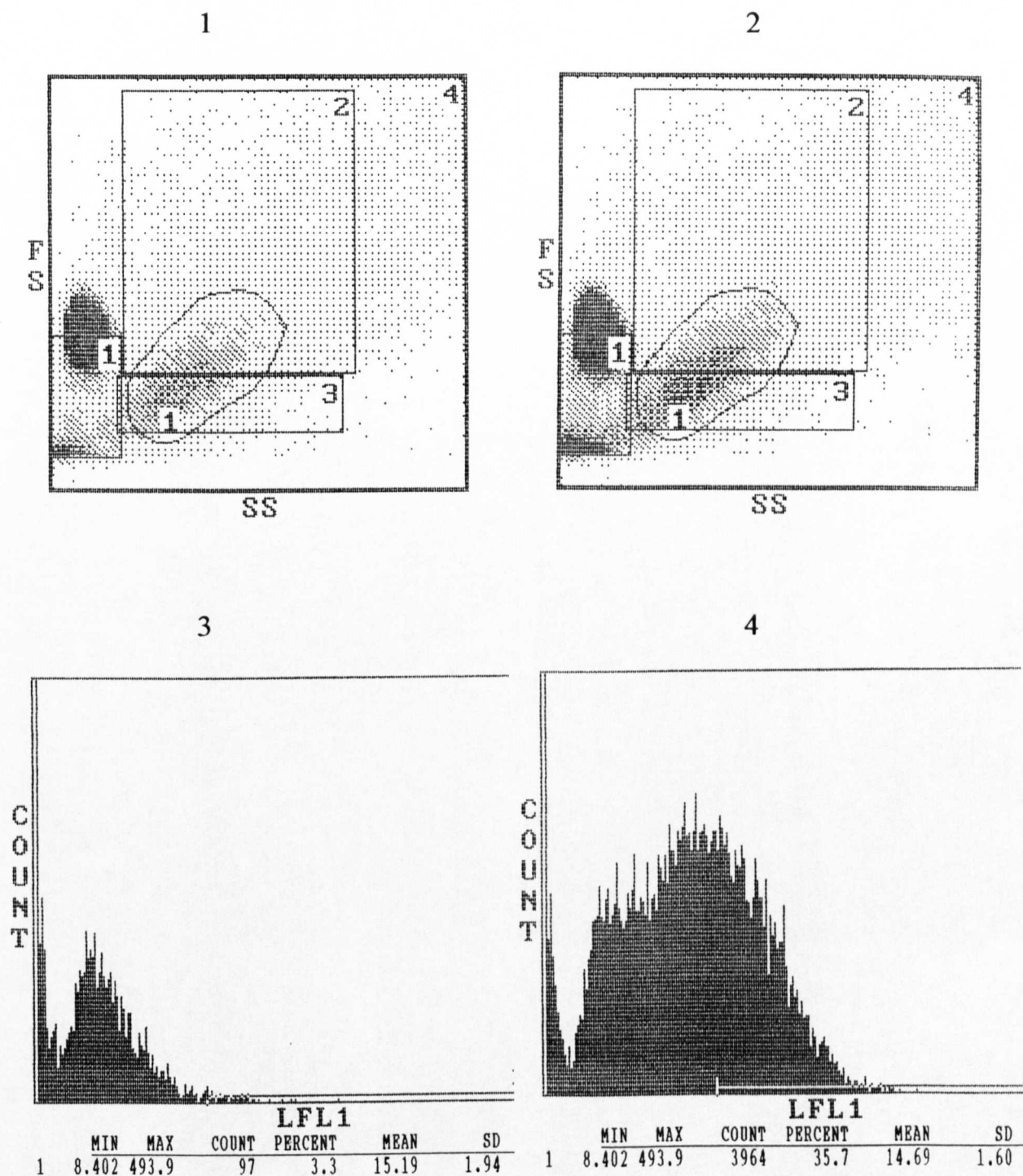


Fig.3.15. Flow cytometric analysis of lung lavage of a guinea pig 24 hours post-infection with CV. The histograms on the left are a control for the sample shown on the right which had been incubated with an anti-granulocyte antibody. 35.7% of cells within the elongated bit-map bound the label compared with 3.3% in the control.

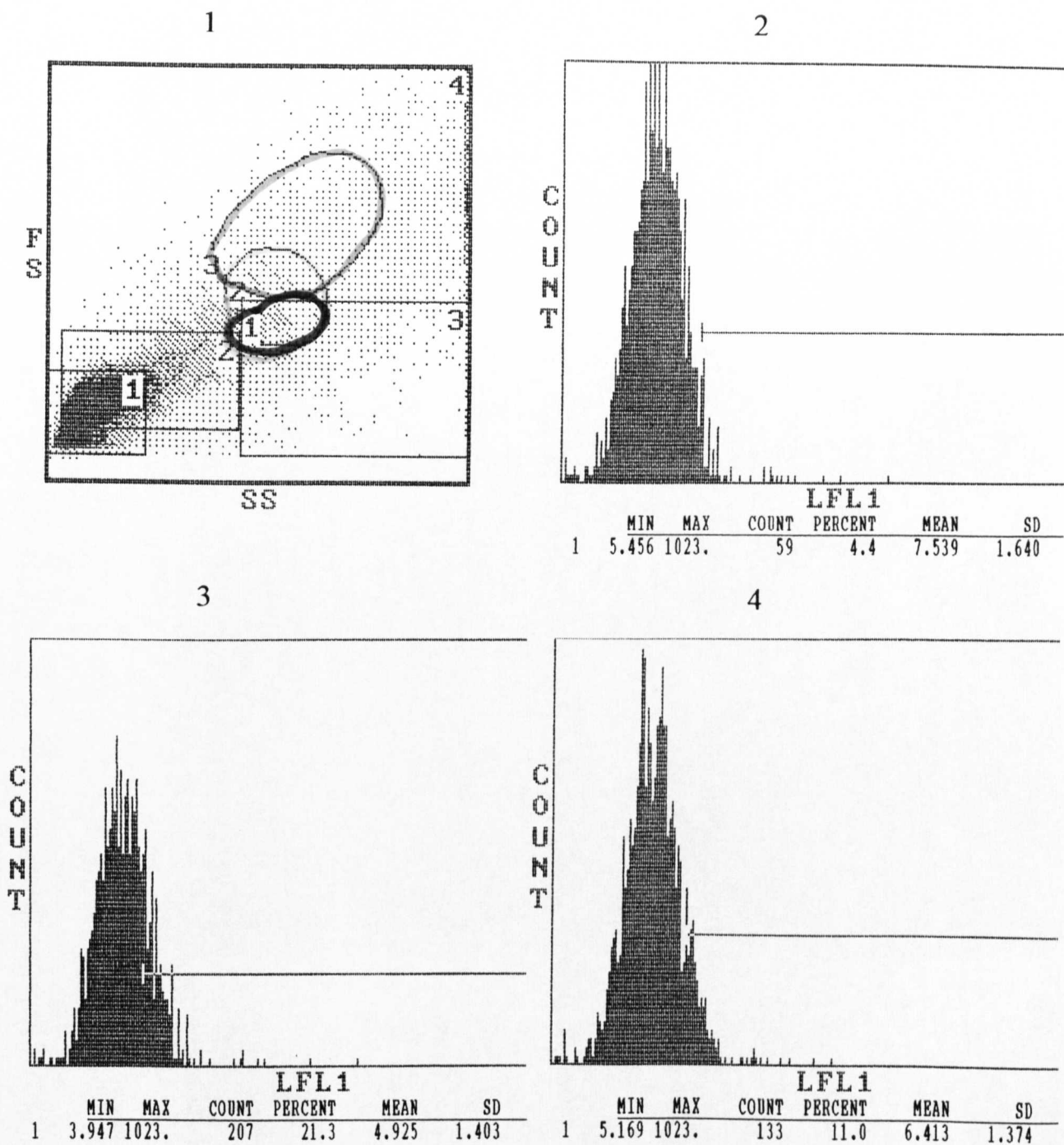


Fig.3.16. Flow cytometric analysis of a guinea pig lung lavage taken 24 hours post-infection with CV. The sample was incubated with anti-HLA-DR antibody. Histogram 1 shows the location of three bit-maps used to identify the macrophage population. Histogram 2 shows fluorescence in bit-map 1 (red), histogram 3 the fluorescence in bit-map 2 (green) and histogram 4 the fluorescence in bitmap 3 (orange). The highest percentage labelling was in bit-map 2 at 21.3% compared with 1.9% in the control (not shown).

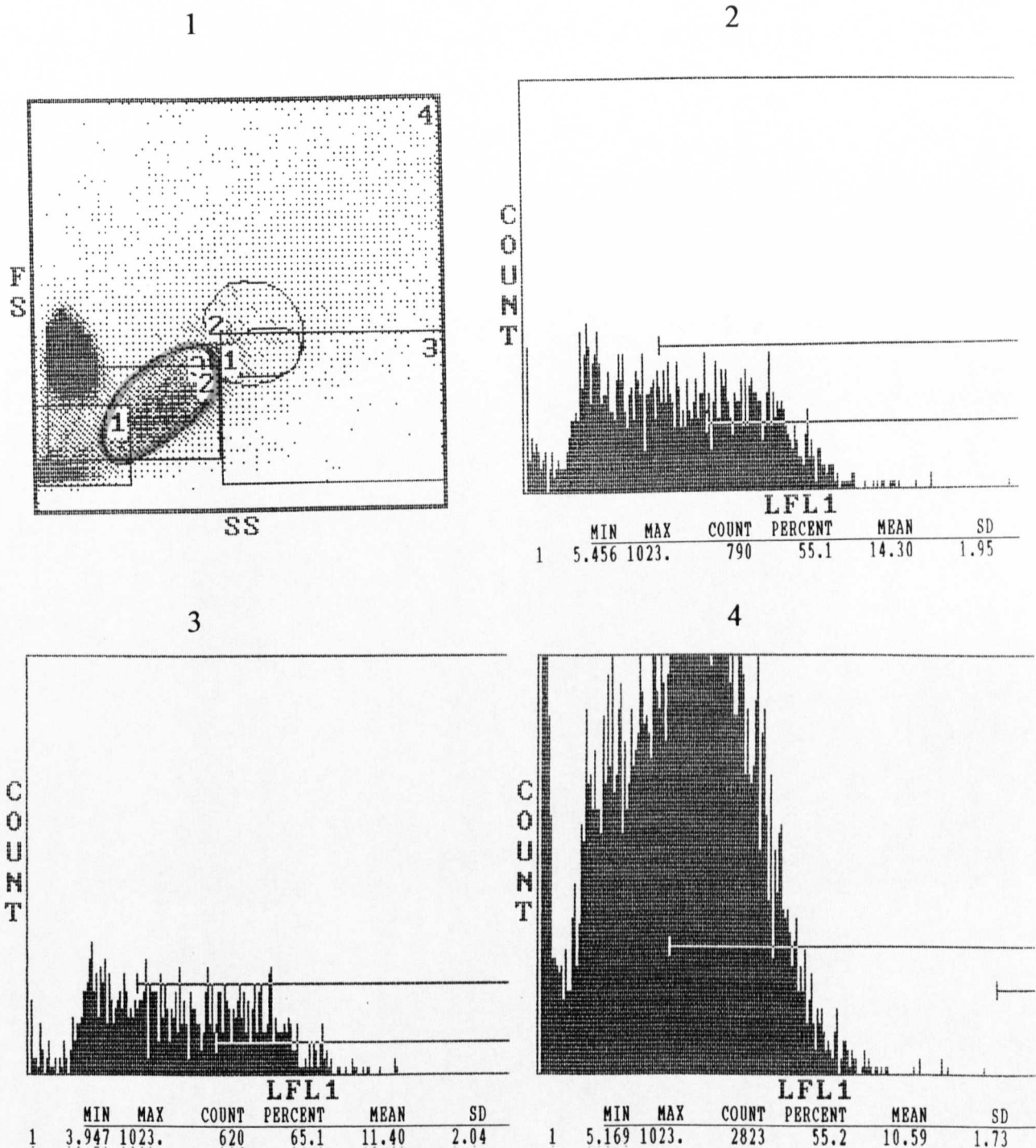


Fig.3.17. Flow cytometric analysis of guinea pig lung lavage 24 hours post infection with CV. The sample is the same as in Fig.3.16 but incubated with an anti-granulocyte antibody. Bit-map 3 in histogram 1 has been moved to locate the PMNL population seen in Fig.3.15. Labelling was greatest in bitmap 3 (orange), 53.4% compared with the control 1.8% (not shown).

the macrophage population was located by using three bit-maps and comparing the degree of HLA-DR labelling in each. The area within bitmap 2 contained the largest percentage of labelled cells but Fig 3.17 shows that this area also contained cells which carried the granulocyte label. Labelling of the cells with an anti *L.pneumophila* LPS antibody is shown in Fig.3.18 and it can be seen that the majority of the label was associated with macrophages although 25% of the events in the PMNL population contained LPS label.

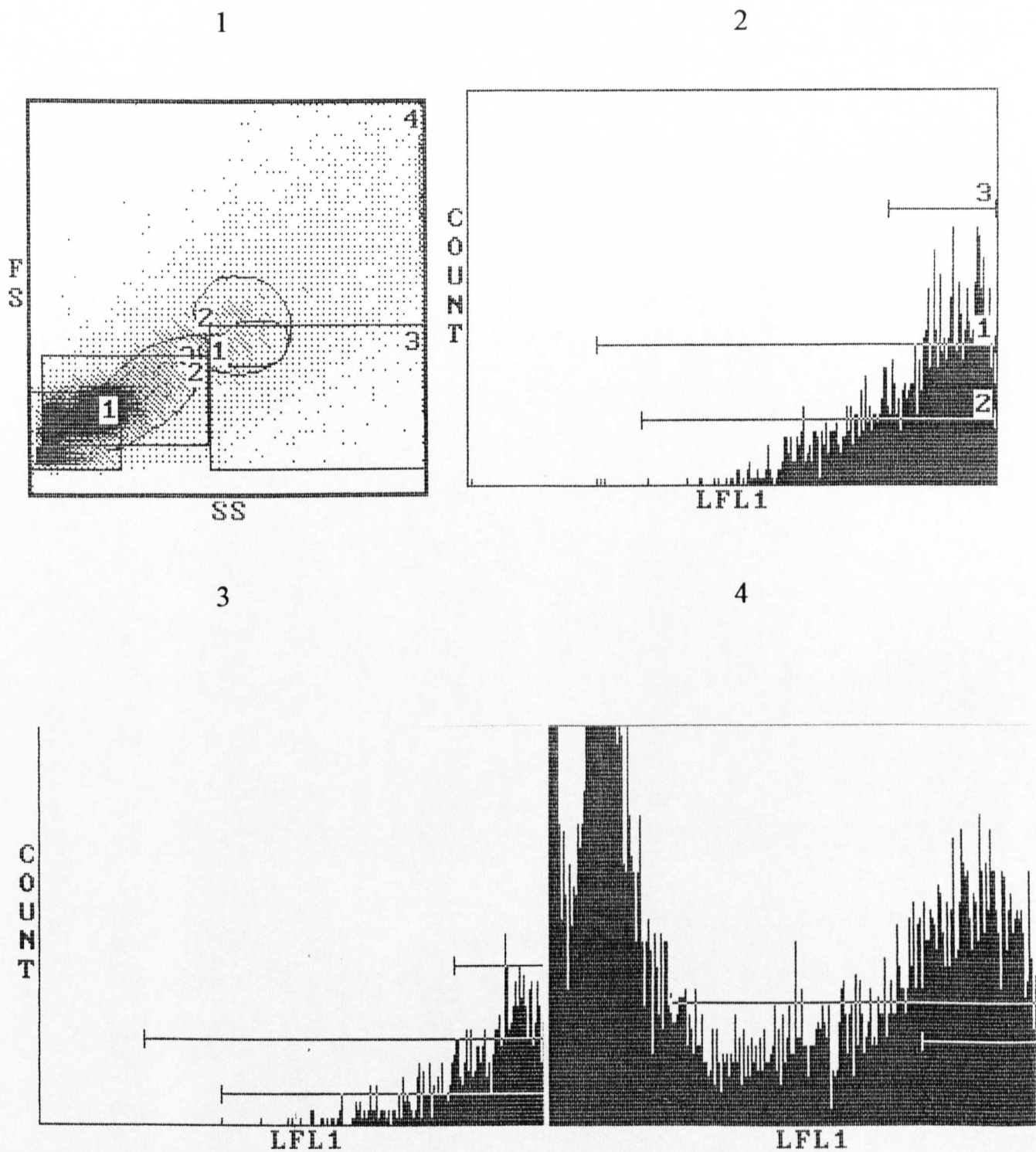


Fig.3.18. Flow cytometric analysis of lung lavage 24 hours post-infection with CV. The sample and histograms as Fig.3.17 but incubated with anti-*L.pneumophila* LPS antibody. The greatest percentage of labelling was in bit-map 2 where the majority of the macrophage population was located.

3.4. DISCUSSION

Studies aimed at elucidating the pathogenic and virulence mechanisms of micro-organisms are most frequently performed *in vitro* where experimental conditions can be carefully controlled without the interference of unknown factors. However there is always a risk in such studies that the laboratory conditions do not successfully mimic those pertaining *in vivo*. Ideally the organism should be examined in the environment appropriate to that which it encounters during disease in its natural host. In the case of LD this environment is the alveolus and its associated cell population and the experiments reported herein were designed to discover interactions of virulent and avirulent strains of *L.pneumophila* with this *in vivo* environment.

Initially the effect of the *in vivo* environment on the bacteria was studied by comparing the surface proteins expressed by legionellae grown on artificial media with those expressed by legionellae multiplying *in vivo*.

L.pneumophila which had been isolated from macrophages during experimental infection were found to express fewer proteins than cells cultured on media when the two were compared by SDS-PAGE and Western blotting. There was also a reduction in the number of protein bands which were recognised by antisera raised against legionellae growing *in vivo*. This suggests that the large numbers of proteins expressed by *L.pneumophila* during artificial culture are not required for intracellular growth. It was not possible to determine, using these methods, whether any of the protein bands on blots of the *in vivo* grown bacteria were unique to the *in vivo* environment. The bands could represent novel proteins expressed *in vivo* which cross react with antibodies against *in vitro* grown *L.pneumophila* or it is possible that such proteins are present on artificially cultured bacteria, but greater expression is induced *in vivo*.

There were distinct changes of staining pattern between media-grown and *in vivo* grown bacteria which merit further comment. A band with molecular weight of approx. 19 kDa was observed which stained only when blots had been incubated with anti-*in vivo* grown Stafford virulent strain. This could represent antibodies raised against the 19 kDa peptidoglycan-associated lipoprotein (Engleberg *et al.*, 1984 & 1991) which is known consistently to elicit a humoral response in guinea pigs (Engleberg, 1993). Interpretation of the Stafford strain blots was limited because there was no possibility of unique *in vivo* induced antigens being seen. Such blots were available for the Corby strain and although no bands unique to these bacteria were identified, the staining was limited to a 19kDa band which could be the antigen described above and to the region of the 36-45 kDa markers. Putative virulence proteins have been described with molecular weights in this region namely legiolysin (39kDa, Wintermeyer *et al.*, 1991) which has haemolytic activity, and zinc metalloprotease or tissue destructive protease which has a molecular weight of 38kDa and has many possible pathogenic activities. There was an obvious lack of reaction on *in vivo* Corby blots in the 29-24kDa region where two of the most widely reported virulence factors (MOMP and mip) should be. It cannot be concluded from these limited experiments that those proteins are not expressed *in vivo* but it is worth noting that the majority of the studies involving the MOMP and mip were performed on BCYE grown *L.pneumophila*.

An interesting result with this experiment was a 24kDa band which was observed only on virulent Stafford strain blots, further investigation may yet prove this to be a virulence associated protein.

Analysis of the proteins produced by *in vivo*-grown bacteria through interpretation of Western blots has certain limitations since there may have been proteins present which were not detected by any of the antisera, either because the specific antibodies were not present or because the process of

immunoblotting destroyed the antigenicity of the proteins. Thus, the Western blot analyses shown in Figs. 3.3 & 3.4, demonstrate that the majority of the antibodies raised against *in vitro* grown bacteria did not react with *in vivo* grown cells, but do not rule out production of novel antigens which were not detectable. It should also be noted that the greater number of protein bands stained with rabbit anti-whole bacteria could be due to non-specific staining of proteins or peptides bound to LPS. This was described in Chapter 2 where an anti-LPS monoclonal antibody appeared to immunolabel protein bands and can be seen in Fig 3.4., lanes 13 and 14 where an anti-LPS antibody produced a similar staining pattern to the rabbit antisera (lanes 1-4).

Given these limitations in the technique a clear difference in the proteins expressed during growth in the *in vivo* and *in vitro* environments was demonstrated.

Similar changes in protein expression were described by Abu-Kwaik *et al.*, (1993) who found that *L.pneumophila* in the presence of a macrophage-like cell line (U937) lost expression of proteins produced on culture media and expressed several novel proteins. That study employed the method of two dimensional electrophoresis which distinguishes proteins by means of their isoelectric potential as well as molecular weight and achieves a better separation of protein moieties. The use of a cell line also facilitated those studies in providing greater quantities of intracellular bacteria. Providing that sufficient quantities of *in vivo* grown legionellae could be obtained with guinea pig cells this could be a more successful technique with which to study these proteins and the stimuli which induce them. There is evidence that many pathogens have the ability to adapt to different environments showing the ability of such organisms to sense changes around them. This is particularly important for pathogens which require exploitation of more than one host. As

mentioned previously *Legionella* species are found widely in water environments yet only a minority of the strains and species cause human disease. It is possible that those found exclusively in the natural environment do not have the regulatory mechanisms to enable the changes in protein expression which allow adaptation to the conditions found *in vivo*. There is also a school of thought that pathogenic legionellae are those which have exploited and multiplied within species of protozoa (Rowbotham, 1986). Similarities between the intracellular behaviour of legionellae within amoebae and macrophages have been shown (Fields, 1993) and it is possible that the ability to synthesise certain proteins could be important. A study by Barker *et al.*, (1993) described differences in surface structures and properties of *L.pneumophila* grown in amoebae and it remains to be seen whether any similarities exist between these and macrophage-grown legionellae. Further investigations of *in vivo* induced proteins would aid the understanding of the virulence mechanisms of LD and possibly provide a further insight into the potential pathogenicity of strains in natural environments.

The experiments described in this chapter were also designed to determine the interaction of *L.pneumophila* with alveolar phagocytes *in vivo*. The early response of guinea pig alveolar phagocytes to airborne infection with virulent and avirulent forms of the Corby strain was compared.

The avirulent strains did not evoke a strong cellular response in the guinea pigs, the cell populations varied little over 30 hours in terms of quantity and type of cell. Macrophages were the dominant cell over most of the experiment but there was no increase in number compared with uninfected animals. Such cellular inactivity suggests that the innate non-immune defence mechanisms were able to deal with the invading bacteria without the elaboration of complex cellular immune mechanisms which are often

described for defence against virulent *L.pneumophila*. Viability of both avirulent strains decreased gradually in the lungs but within the time-course of the experiment they were not completely eliminated. The CAC avirulent form, also decreased in viability with time in the *in vitro* assay described in Chapter 1. The behaviour of CA differed between the *in vitro* assay and following challenge of guinea pigs. Viability was retained in the *in vitro* assay but not *in vivo*. An explanation could be the efficiency of killing by macrophages where CAC was effectively controlled by the phagocytes but CA was less susceptible. This could be seen when the intra- and extracellular viability of the strains were compared. The predominance of viable CAC changed from being intracellular to extracellular suggesting that intracellular defence mechanisms were effective but over most of the experiment there were more viable CA within cells than without. The decrease in overall viability of CA in guinea pigs would probably have been mediated by both macrophages and PMNLs. An *in vivo* study of an avirulent strain of *L.pneumophila* Philadelphia 1 (NCTC 11192) found the organism to be predominantly extracellular with intracellular bacteria distributed equally between macrophages and PMNLs (Jepras *et al.*, 1985). This suggests that macrophages and PMNLs do not support the growth of avirulent strains *in vivo*. Thus, results obtained in the *in vitro* macrophage assay, although indicative of virulence do not necessarily mirror the situation *in vivo*.

The Corby virulent strain induced a marked inflammatory response as demonstrated by an increase in PMNLs which corresponded with a decrease in viability in the early stages of infection. This may reinforce the fact that virulent *L.pneumophila* strains are susceptible to killing by these cells. However, since high levels of PMNLs were maintained, the eventual continued multiplication of CV suggests that any inhibition by PMNLs was overcome. Protection within macrophages may have been a

factor since CV was located predominantly intracellularly but the large numbers of viable extracellular bacteria suggests that there was an inhibition of PMNLs in some way. Rechnitzer & Kharazmi (1992) have shown that *L.pneumophila* protease inhibited PMNL chemotaxis and this will be investigated and discussed further in the following chapter. Clearly the recruitment to the lung of these cells was not affected but it is possible that a more local action of the protease prevented the PMNLs from efficiently phagocytosing the bacteria. Resistance to the killing mechanisms of the PMNLs is another possibility since *L.pneumophila* has been shown to produce substances which can inhibit the toxic oxidative responses of these cells. These are the protease mentioned above (Sahney *et al.*, 1993) and a low molecular weight toxin (Friedman *et al.*, 1982, Lochner *et al.*, 1985). Phagocytosis of the virulent Corby in this study was shown by the fact that 25% of the cells in the PMNL population were labelled for LPS (Fig.18) but whether this represented viable or killed bacteria was not determined. Jepras *et al.*, (1985) found, using the same virulent strain in a similar study that, at 24 hours post-infection, 10% of the total viable bacteria were located within PMNLs. To conclude, a proposal of the role of PMNLs in control of infection with *L.pneumophila* would be that even though virulent strains are susceptible to killing by these cells and do not replicate within them, the production of enzymes and toxins by the bacteria multiplying within macrophages reduces the toxicity of PMNLs. Thus, in the absence/deficiency of PMNLs for example in disease, the virulence of *L.pneumophila* is increased and enhancement of PMNL function has proven effective in the control of human LD (Hollander *et al.*, 1991).

Further analysis of the eosinophil population was performed since these cells can be confused with PMNLs on stained cell smears. Histopathological examination of the lungs of guinea pigs infected with virulent and avirulent strains revealed that there were changes in the numbers of these cells over

the course of this experiment. There was little difference in the numbers between virulent and avirulent infected lungs. The involvement of eosinophils in the pathology of LD has not been previously noted and since the Pathologist who examined the sections described their distribution as being mainly peribronchial it is probable that direct interaction of eosinophils with legionellae in the alveoli is not a feature of the disease. These findings do, however illustrate the importance of *in-situ* examination of events occurring *in vivo*.

These results agree in general with those of Jepras *et al* (1985) in their comparison of the Corby (virulent) strain with the Philadelphia 1 (avirulent) strain although some differences exist. In the current studies the peak of PMNL influx occurred 16 hours before the peak reported by Jepras *et al*. This could be explained by the higher initial dose of Corby in the present study which was approximately 100 fold more than that of Jepras *et al*. The avirulent strain used in that study persisted in guinea pig lungs 14 days following aerosol challenge. The differences between that strain and the avirulent strains in these experiments show that conversion of strains by multiple passage on artificial media is not a straightforward, one component process and this results in subtle differences in the responses of the host to challenge with different avirulent strains. Such variations could be partially responsible for the lack of clear evidence concerning the virulence mechanisms of LD due to the number of different strains of *L.pneumophila* which have been studied.

The analysis by flow cytometry of the variations in cell populations yielded little more information than microscopy and bacteriology due to the various difficulties described in the results section. The problems could have been partially overcome by more extensive testing of reagents and familiarisation of the cell distributions using uninfected lavages. To compensate for the variation in the numbers of cells obtained from animals and the alterations in

cell size and granularity over the course of the disease would require more guinea pigs per time point. The overlap of the cell populations with each other and the wide spread of the macrophage populations as seen by the low percentages of events labelled in each bit-map (maximum obtained was 50% labelling of granulocytes) was probably the limiting factor to the success of these experiments. Flow cytometry is most successful in well defined cell populations where variations in the quantity and types of cells can be reliably compared. The use of flow cytometry to examine the responses of phagocytic cells to legionellae and other bacteria Bass *et al* (1983) has been proven with cells cultured *in vitro* but such studies were beyond the scope of this investigation which sought to examine events occurring *in vivo*. The reactivity of monoclonal antibodies specific for human and mouse lymphocyte markers with guinea pig cells was an interesting finding which indicated a similarity of these molecules within mammalian species.

In summary the work described in this chapter has demonstrated that *L.pneumophila* growing within guinea pig macrophages exhibits differing surface proteins to legionellae grown on artificial culture media and that the host (guinea pig) response to virulent and avirulent bacteria differs from that seen with macrophages isolated in tissue culture. The conclusion therefore is that whilst *in vitro* studies serve a purpose to identify potential virulence factors the relevance of these to the disease being studied must be confirmed by examination of the organism within the environment of the natural disease.

This philosophy is continued in the next Chapter which investigates the role of a pathogenic factor to which has been attributed many disease-causing properties but has received conflicting reports concerning its importance in the disease as a whole.

CHAPTER 4

Tissue destructive protease of *Legionella pneumophila*

4.1. INTRODUCTION

The main source of energy for *Legionella* species is provided by extracellular proteases which are required for cleavage of proteins and peptides to amino acids. Not long after the first descriptions of the pathology of LD, proteases, along with extracellular toxins became the prime candidates to explain the pulmonary and extra pulmonary lesions. Following the discovery that several human proteins were degraded by *L.pneumophila* (Muller, 1980), interest in proteases grew and large number of extracellular enzyme activities have since been described (Muller, 1980 & 1981; Thompson *et al.*, 1981; Berdal & Fossum, 1982; Berdal *et al.*, 1982; Baine *et al.*, 1985; Conlan *et al.*, 1986; Gul'nik *et al.*, 1986; Rdest *et al.*, 1991). Conlan *et al.*, (1986) showed that of six discrete protease activities purified from *L.pneumophila* broth culture supernatants, only one caused necrosis in the skin of guinea pigs. This tissue destructive protease (TDP) was a zinc metalloprotease which has since been studied by a number of workers (Dreyfus & Iglewski, 1986, Quinn & Tompkins, 1989, Blander & Horwitz, 1989). This enzyme, has been shown to be at least partially responsible for the pulmonary damage seen in experimental LD in guinea pigs. When purified TDP was instilled intranasally in guinea pigs it produced lesions pathologically similar to those seen in human and experimental LD (Baskerville *et al.*, 1986). Quantities of TDP sufficient to cause such lesions were detected *in vivo* by an ELISA technique in lung homogenates of *L.pneumophila* infected guinea pigs (Conlan *et al.*, 1988a) and were

localised in the lung tissue closely associated with the bacteria and pulmonary damage (Williams *et al.*, 1987).

TDP acts on a variety of protein substrates (Conlan *et al.*, 1986) and has been shown to degrade human serum proteins (Muller, 1980 & 1983), inactivate human α -1-antitrypsin (Conlan *et al.*, 1988b) and inhibit human neutrophil chemotaxis (Rechnitzer & Kharazmi, 1992). Cytotoxic activities of *L.pneumophila* have been attributed to this protease and genetic studies have confirmed this enzyme to be responsible for the cytolysis of CHO cells and haemolysis in vitro (Quinn & Tompkins, 1989; Keen & Hoffman, 1989).

Pseudomonas aeruginosa and *Serratia marcescens* secrete extracellular proteases which have similar properties to TDP. The enzymes are known to be virulence and pathogenicity factors for certain diseases caused by these bacteria (Holder & Haidaris, 1979; Woods *et al.*, 1982; Lyster & Kreger, 1983). Action on a number of substrates by these enzymes is involved, one example of which is the inactivation of immunoglobulins IgG and IgA (Holder & Wheeler, 1984; Molla *et al.*, 1986; Heck *et al.*, 1990). Degradation of human IgG, IgA and IgM by legionellae grown in the presence of serum has been demonstrated by Muller (1983) but the effects of purified *Legionella* enzymes on immunoglobulins are unknown and were investigated.

A protease with the same properties as TDP has been purified from *L.pneumophila* Philadelphia 1 strain and named major secretory protein (MSP). MSP is believed to have a role in cell-mediated and protective immunity to experimental LD indicated by lymphocyte proliferation (Breiman *et al.*, 1987; Blander & Horwitz, 1989). However, Conlan (1987) found that immunisation with TDP did not protect guinea pigs from lethal challenge with the CV, despite giving rise to high titre antibodies. In support of the latter study, Rechnitzer & Kharazmi, (1992)

demonstrated that *L.pneumophila* TDP may repress the host response by inhibition of neutrophil chemotaxis and of the ability of monocytes to kill intracellular *Listeria*.

Pseudomonas elastase and alkaline protease have been implicated in modulation of cellular immunity in a number of ways including inhibition of PMNL function (Kharazmi *et al.*, 1986) and chemotaxis (Kharazmi *et al.*, 1984), and inhibition of natural killer cells (Pederson *et al.*, 1987). The enzymes are known to inactivate cytokines such as gamma interferon (IFN γ) (Horvat *et al.*, 1989) and interleukins (IL) (Theander *et al.*, 1988), suggesting a mechanism for the inhibition of immune cell function. It is possible that the inhibitory effects of *L.pneumophila* protease described by Rechnitzer & Kharazmi (1992) were also mediated via cleavage of cytokines. The effect of TDP on IFN γ and IL2 was determined in the present study.

Natural lung defences include protease inhibitors which protect against damage by endogenous proteases. A high molecular weight glycoprotein, alpha-2-macroglobulin (α 2M) has been shown to inhibit most endopeptidases non-specifically (Barrett, 1973). This is a major plasma protein but is also synthesised and secreted by cultured peripheral blood monocytes (Hovi *et al.*, 1979), fibroblasts (Mosher & Wing, 1976), embryonic lung cells (Mosher *et al.*, 1977) and human alveolar macrophages (White, 1980). The latter studies suggested a role for the inhibitor in defence of the lung against potentially harmful proteases. This mechanism could also be involved in protection against proteases produced by bacteria which cause bronchopneumonia's. Inactivation of TDP by α 2M *in vivo* could possibly protect the host against such lung damage. *Serratia marcescens* and *Pseudomonas aeruginosa* proteases are able to inactivate α 2M thereby evading a potential host defence mechanism (Molla *et al.*, 1986; Horvat *et al.*, 1989). The following

studies were designed to investigate the interaction of TDP with α 2M and determine the implications of this for LD pathogenesis.

The significance of the *in vitro* observations of TDP activity depends on demonstration of the enzyme being produced *in vivo*. The *in vivo* production of protease has been demonstrated and quantified by an ELISA in the lungs of guinea pigs with experimental LD in quantities sufficient to cause some of the effects seen *in vitro* (Conlan *et al.*, 1988a). Production of the enzyme by *L.pneumophila* multiplying intracellularly has not been directly demonstrated. Assays based on intracellular growth of *L.pneumophila* within guinea pig macrophages were used in this study to investigate protease production. However, immunological detection of TDP *in vivo* does not prove that the enzyme is capable of the activities described above. To determine whether the enzyme detected *in vivo* was functional, protease was purified from infected guinea pig lung washouts and tested for enzymatic function.

Szeto & Shuman (1990) have described a transposon mutant of *L.pneumophila* which was defective in secretion of the major secretory protein. Blander *et al* (1990) found that aerosols of this mutant were as lethal for guinea pigs as the MSP-producing parent strain. This would appear to conflict with studies in which numerous pathogenic properties have been attributed to the protease. In this chapter, the mutant and its parent strain were compared with the Corby strain in terms of protease production and in the guinea pig model in an attempt to explain this anomaly.

4.2. MATERIALS AND METHODS

4.2.1. TDP PURIFICATION

TDP was purified from broth (YEB) culture supernatants of *L.pneumophila* Corby virulent strain as described by Conlan *et al.* (1987).

4.2.2. Alpha-2-MACROGLOBULIN PURIFICATION

A preparation of $\alpha 2M$ was first obtained from a commercial source (Boeringer) but this showed little inhibitory capacity which then rapidly diminished, so the inhibitor was purified from human plasma by the method of Barrett (1981). Briefly, human plasma was obtained from a healthy adult volunteer. Lipoproteins were removed by polyethylene glycol 6000 (PEG) precipitation, the remaining proteins precipitated out by PEG and separated by Sepharose 6B gel filtration with phosphate buffered saline (PBS) pH 7.4 as eluent at a flow rate of 20 ml hour⁻¹. Fractions containing $\alpha 2M$ were identified by SDS-PAGE and a specific inhibition assay described below.

4.2.3. EFFECT OF TDP ON IFN γ and IL-2

Purified TDP at concentrations similar to those detected *in vivo* (20 μ g ml⁻¹, Conlan *et al.*, 1988a) was incubated with purified human IFN γ and IL-2 (natural and recombinant, Sigma) at 37 $^{\circ}$ C for various times and the reaction mixtures analysed by SDS-PAGE using Excel gels were stained with Coomassie blue, as described in Chapter 2. Controls of IFN γ and IL-2 incubated with buffer were included.

4.2.4. EFFECT OF TDP ON IgG AND IgA

Purified TDP at $72 \mu\text{g ml}^{-1}$ was incubated with $100 \mu\text{g ml}^{-1}$ human IgG and IgA (Sigma) for 5 hours at 37°C and the reaction mixtures analysed by SDS-PAGE as above.

The effect of TDP on the Fc- and antigen-binding regions of IgG and IgA was determined using Enzyme-linked immunosorbent assays which were dependent on the binding of the immunoglobulins in one or other of these regions. The assays for human IgA were those described by Ashworth *et al* (1982) and involved estimates of total IgA (Fc bound) and of IgA with a specificity for *Bordetella pertussis* antigens. A rabbit assay of IgG specific for *L.pneumophila* LPS was that described by Conlan and Ashworth (1986)

4.2.5. ASSAY OF INHIBITION OF THERMOLYSIN BY $\alpha 2\text{M}$

This assay was used to estimate the capacity of $\alpha 2\text{M}$ to inhibit the activity of thermolysin as described by Barrett (1981). Thermolysin activity was measured spectrophotometrically as the absorbance due to the release of dye from either Azocoll or Hide powder azure protein substrates (Sigma). Percentage inhibition of thermolysin by $\alpha 2\text{M}$ was calculated by comparing the residual thermolysin activity following incubation with $\alpha 2\text{M}$ with the activity of thermolysin incubated without inhibitor.

4.2.6. INHIBITION OF TDP BY $\alpha 2\text{M}$

The inhibition of TDP by $\alpha 2\text{M}$ was determined in a similar assay to that described above because TDP also releases dye from Azocoll. A standard amount of TDP ($1\mu\text{g}$) was incubated with various, increasing quantities of $\alpha 2\text{M}$. After an incubation period of 10 min at 37°C the activity of TDP was measured. The percentage inhibition of TDP by $\alpha 2\text{M}$ was calculated

by comparing this activity with that of TDP in a control where inhibitor was replaced by buffer (Tris HCl pH 7.2). Incubation with Azocoll was for 30min at 37°C after which absorbance was read on a Titretek Multiscan plate reader at 492nm.

4.2.7. PROLONGED INCUBATION OF TDP WITH α 2M

To investigate changes in TDP activity on prolonged incubation with α 2M the protease was reacted with the inhibitor at a molar ratio such that TDP had a residual activity of approximately 10% of the TDP-only control. Incubation was continued for various lengths of time up to 28 hours. After each time substrate was added and the residual activity of TDP determined.

4.2.8. DEGRADATION OF α 2M BY TDP

The TDP inhibition assay described above was used to determine the molar ratio at which TDP was completely inhibited by α 2M. At this molar ratio, mixtures of inhibitor and protease were incubated for various lengths of time then examined by one of the following procedures:

1. Analysis by SDS-PAGE

Samples of α 2M which had been incubated with buffer, TDP, or thermolysin for 0, 0.5, 1 and 1.5 hours were separated in horizontal, linear 5% acrylamide gels as described in Chapter 1.

2. Thermolysin inhibition assay

Following incubation with TDP or buffer a quantity of thermolysin was added to the mixture and incubation continued for 10 min after which free thermolysin activity was measured with hide powder as substrate.

4.2.9. PROTECTION STUDIES

To investigate the possibility of a therapeutic role for $\alpha 2M$ in Legionnaires' disease guinea pigs treated with purified $\alpha 2M$ were challenged with an aerosol of *L.pneumophila*. In two separate experiments two groups of 6 female Dunkin-Hartley guinea pigs weighing 300-350g were exposed to *L.pneumophila* Corby strain as described in Chapter 2. Both groups were given a 5LD₅₀ dose. In experiment A, one group was given a single intraperitoneal injection of 40mg human $\alpha 2M$ 6h post infection and in experiment B, two intraperitoneal injections of 40 mg each were given 4 and 24 hours post infection. In both experiments control groups were given PBS in place of the $\alpha 2M$.

The animals were observed for sickness and temperatures were taken throughout the course of the illness.

Serum levels of $\alpha 2M$ following intraperitoneal treatments of an uninfected guinea pig were quantified by a commercial immunodiffusion assay (Behring), consisting of agar plates into which defined quantities of antibodies to $\alpha 2M$ were incorporated.

4.2.10. IMMUNOCHEMICAL DETECTION OF TDP

TDP was detected by an antigen capture ELISA based on rabbit anti TDP IgG raised against purified TDP. The specificity of this antibody and the ELISA has been determined and described previously (Conlan *et al.*, 1988a).

4.2.11. INTRACELLULAR PRODUCTION OF TDP IN ALVEOLAR MACROPHAGES

Guinea pig alveolar macrophages were maintained in tissue culture and infected with *L.pneumophila* strains as described in Chapter 2 and incubated for 24 hours. Enumeration of bacteria by viable counts was

performed in culture supernatants and macrophage lysates immediately following removal of extracellular bacteria and after 24 hours incubation using the methods described in Chapter 2. These samples were also assayed for TDP using the ELISA.

4.2.12. IMMUNOGOLD LABELLING OF TDP

Guinea pig alveolar macrophages which had been incubated with *L.pneumophila* for 24 hours were harvested and processed for transmission electron microscopy as described by Williams *et al.*, (1987). Ultrathin sections of infected cells were immunogold labelled using an indirect method (Williams *et al.*, 1987) with rabbit anti-protease or rabbit anti-LPS as primary antibodies followed by goat anti-rabbit IgG gold (10nM, Biocell).

4.2.13. *IN VIVO* ACTIVITY OF TDP

Guinea pigs were given a lethal aerosolised dose of *L.pneumophila* as described in Chapter 2. After 3 days their lungs were washed out post mortem and cells were disrupted by sonication. Cell debris and bacteria were removed and the supernatant equilibrated in 25 mM Tris-HCl pH 7.0 and applied to a FPLC Mono Q anion exchange column (Pharmacia) equilibrated with the same buffer. Proteins were eluted off the column with a 0-0.5M NaCl gradient at a flow rate of 1 ml min⁻¹ and fractions were assayed for TDP by ELISA. Fractions containing TDP were pooled and concentrated by pressure dialysis over a low protein absorption ultrafiltration membrane (Amicon) with a molecular weight cut off of 10000. The concentrate was assayed by ELISA, then added to wells in an agar plate containing 1% sodium caseinate. The plate was incubated at 37°C for several hours and examined for precipitation of the casein.

4.2.14. COMPARISON OF PROTEASES FROM PHILADELPHIA 1 AND CORBY STRAINS

The non-protease producing mutant strain of Philadelphia 1 (2102) and its parent (2029) were a generous gift from Dr. H. Shuman, New York. These strains were grown in YEB and the bacteria-free supernatants were concentrated by pressure dialysis, assayed for TDP activity by ELISA and casein precipitation and compared with TDP purified from CV.

4.2.15. GROWTH OF STRAINS 2102 AND 2029 IN GUINEA PIG MACROPHAGES

The mutant and parent strains were tested in the intracellular growth assay as described in Chapter 2.

4.2.16. VIRULENCE OF STRAINS 2102 AND 2029

The two strains were plated on BCYE agar and suspensions of the growth made in distilled water. These suspensions were aerosolised and administered to guinea pigs as described in Chapter 2. An LD₅₀ experiment was performed and, in a separate experiment to monitor progress of the infection, infected guinea pig lungs were removed immediately after challenge and at daily intervals thereafter for viable counts, TDP assay and histopathology.

4.2.17. EFFECT OF CULTURE SUPERNATANT ON GUINEA PIGS

Concentrated broth culture supernatants of 2102 and 2029 were instilled intranasally into guinea pigs as described by Conlan *et al* (1987). The animals were observed for signs of illness and lungs examined macroscopically and by histopathological techniques

4.3. RESULTS

4.3.1. EFFECT OF TDP ON IFN γ

The result of incubating 24 $\mu\text{g ml}^{-1}$ TDP for 5 hours with various quantities of human, natural and recombinant IFN γ is shown in Fig.4.1. This quantity of enzyme degraded recombinant IFN γ completely but did not appear to degrade the natural form. Fig.4.2 shows that degradation of the recombinant IFN γ by only 12 $\mu\text{g TDP ml}^{-1}$ occurred after 4 hours incubation.

4.3.2. EFFECT OF TDP ON IL2

Incubation of IL2 with TDP resulted in the appearance of a minor protein band on the gels but did not appear to alter the dominant protein (Fig.4.3)

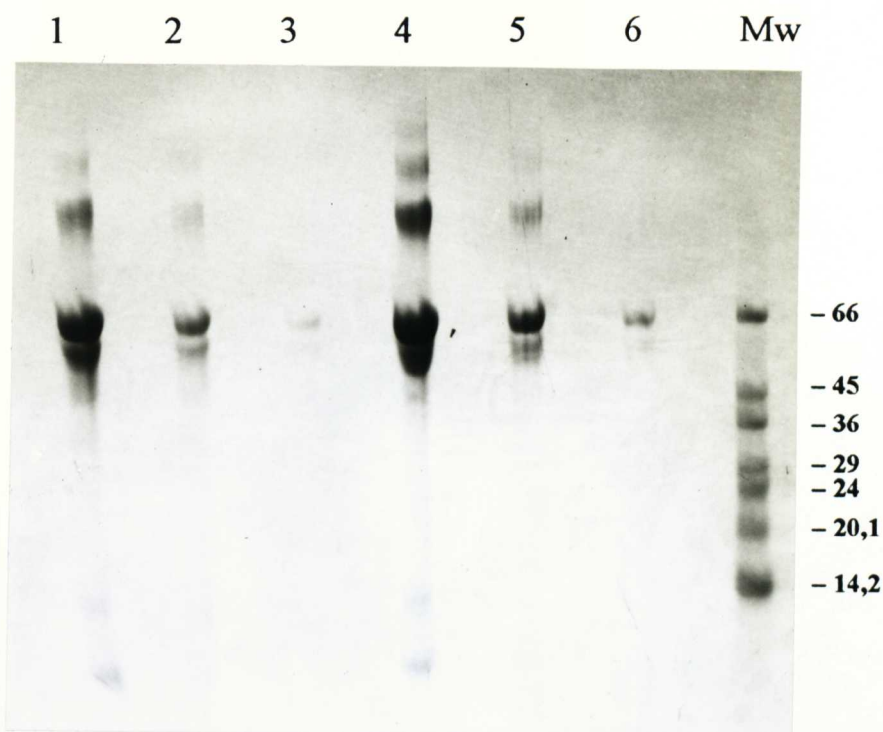
4.3.3. EFFECT OF TDP ON IgG AND IgA

No degradation of either immunoglobulin following incubation with TDP was apparent by SDS-PAGE (Fig.4.4a) however the ELISA results in Fig.4.4b demonstrate that the enzyme affects the ability of rabbit anti-LPS IgG to bind to its specific antigen in an ELISA. Incubation with TDP had no effect on the human IgA in an ELISA measuring total IgA which is indicative of Fc binding and no difference was seen in the ability of human IgA to bind to *Bordetella pertussis* antigens following TDP treatment.

4.3.4. INHIBITION OF TDP BY α 2M

TDP activity was inhibited by α 2M in a concentration dependent manner. Fig.4.5 shows percentage inhibition of TDP by various quantities of α 2M. The activity of 1 μg of enzyme was completely inhibited by 14 μg of

A.



B.

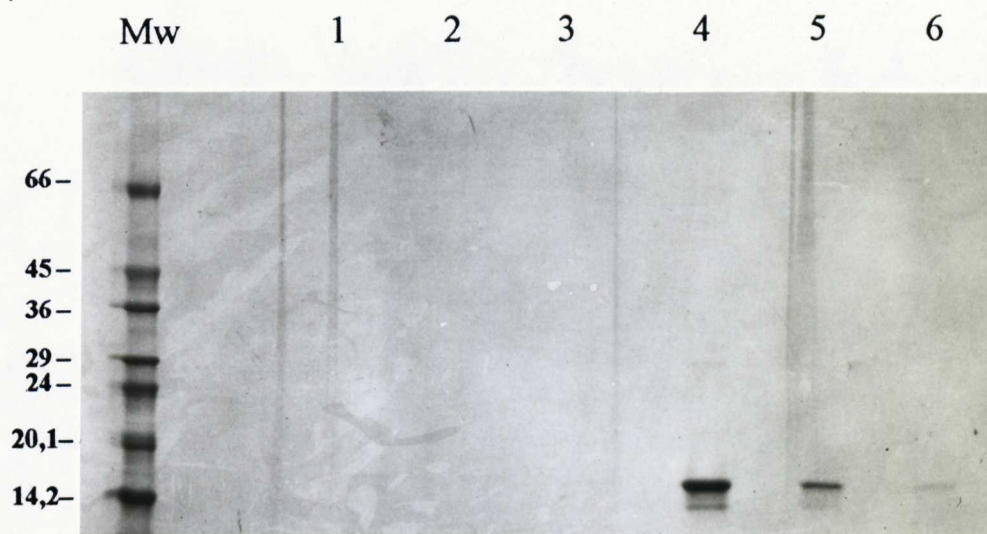


Fig.4.1 Effect of 5 hours incubation with 24µg TDP or buffer on IFN γ .

A. Human natural IFN γ

1, 1×10^4 U IFN γ + TDP;

2, 2×10^3 U IFN γ + TDP;

3, 200 U IFN γ + TDP;

4, 1×10^4 U IFN γ + buffer;

5, 2×10^3 U IFN γ + buffer;

6, 200 U IFN γ + buffer.

B. Human recombinant IFN γ . 1-6 as above but IFN γ at 2×10^5 U, 4×10^4 U and 4×10^3 U respectively.

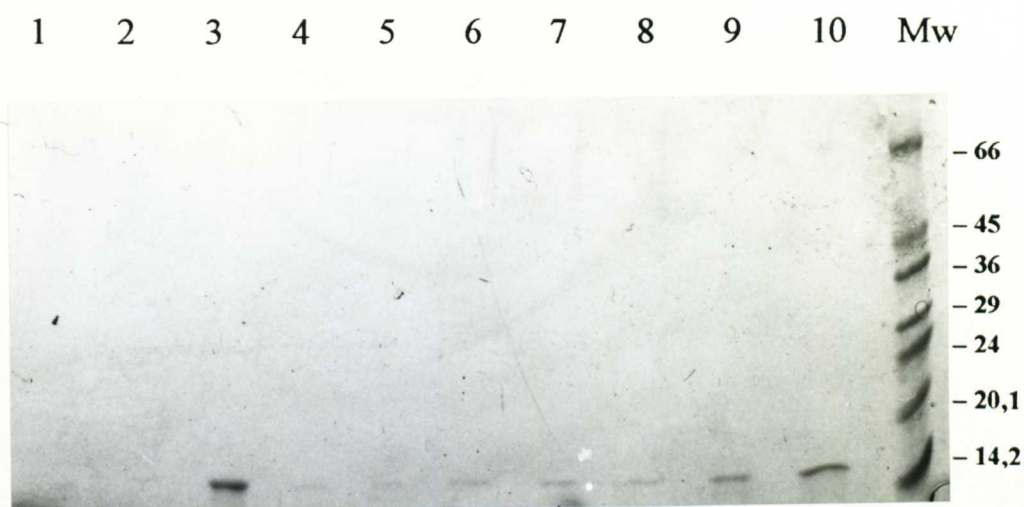


Fig.4.2 Effect of incubation of 12 and 24 μg TDP with 4×10^4 U recombinant IFN γ for various lengths of time.

- 1, IFN γ + 24 μg TDP, 6 hours;
- 2, IFN γ + 12 μg TDP, 6 hours;
- 3, IFN γ + buffer, 6 hours;
- 4, IFN γ + 24 μg TDP, 4 hours;
- 5, IFN γ + 12 μg TDP, 4 hours;
- 6, IFN γ + 24 μg TDP, 2 hours;
- 7, IFN γ + 12 μg TDP, 2 hours;
- 8, IFN γ + 24 μg TDP, 1 hour;
- 9, IFN γ + 12 μg TDP, 1 hour;
- 10, IFN γ + buffer, 1 hour.

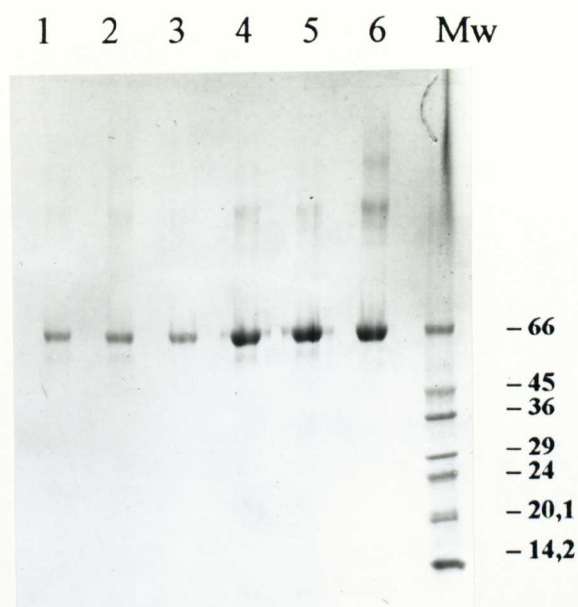
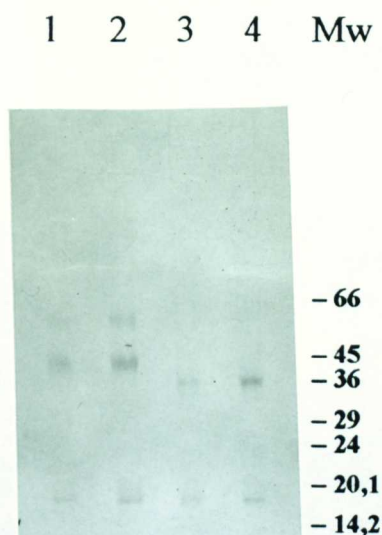


Fig.4.3. Effect of incubating human, natural (n) and recombinant (r) IL2 with TDP.

- 1, IL2r + 72 µg TDP;
- 2, IL2r + 24 µg TDP;
- 3, IL2r + buffer;
- 4, IL2n + 72 µg TDP;
- 5, IL2n + 24 µg TDP;
- 6, IL2n + buffer.

A.



B.

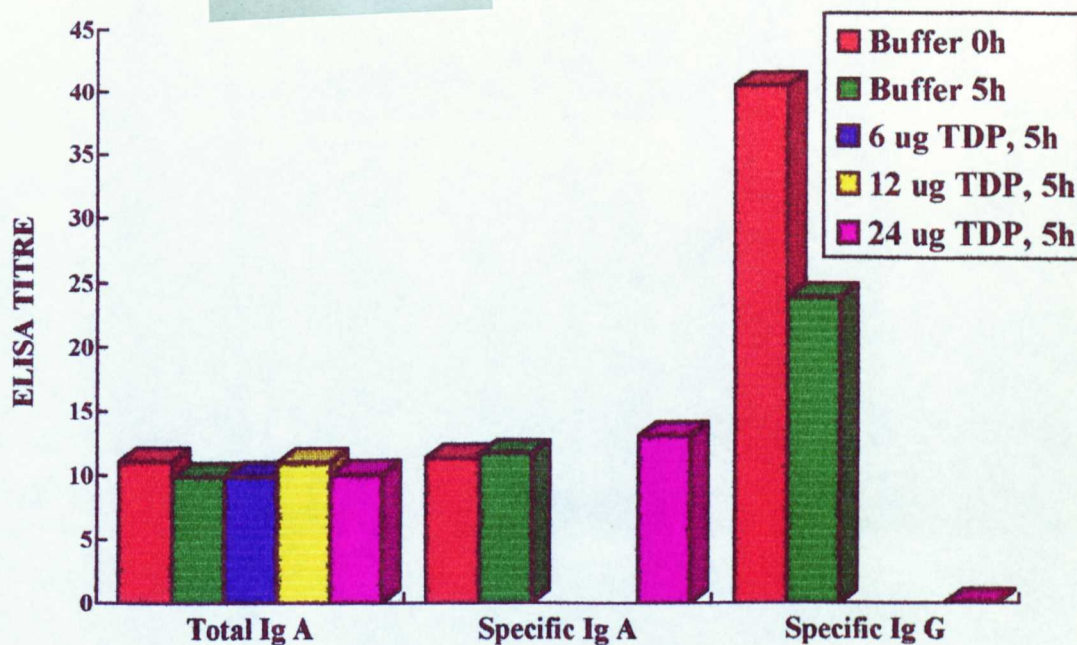


Fig.4.4 Effect of incubating TDP with purified immunoglobulins.

A. SDS PAGE of human IgG and IgA following incubation for 5 hours with $24 \mu\text{g ml}^{-1}$ TDP.

1, IgG + buffer; 2, IgG + TDP; 3, IgA + buffer; 4, IgA + TDP.

B. ELISA titre of immunoglobulins before and after incubation with TDP or buffer in assays measuring total immunoglobulin (Fc binding) or antigen-specific immunoglobulin. Note that specific IgA and IgG not tested with TDP at 6 and 12 μg .

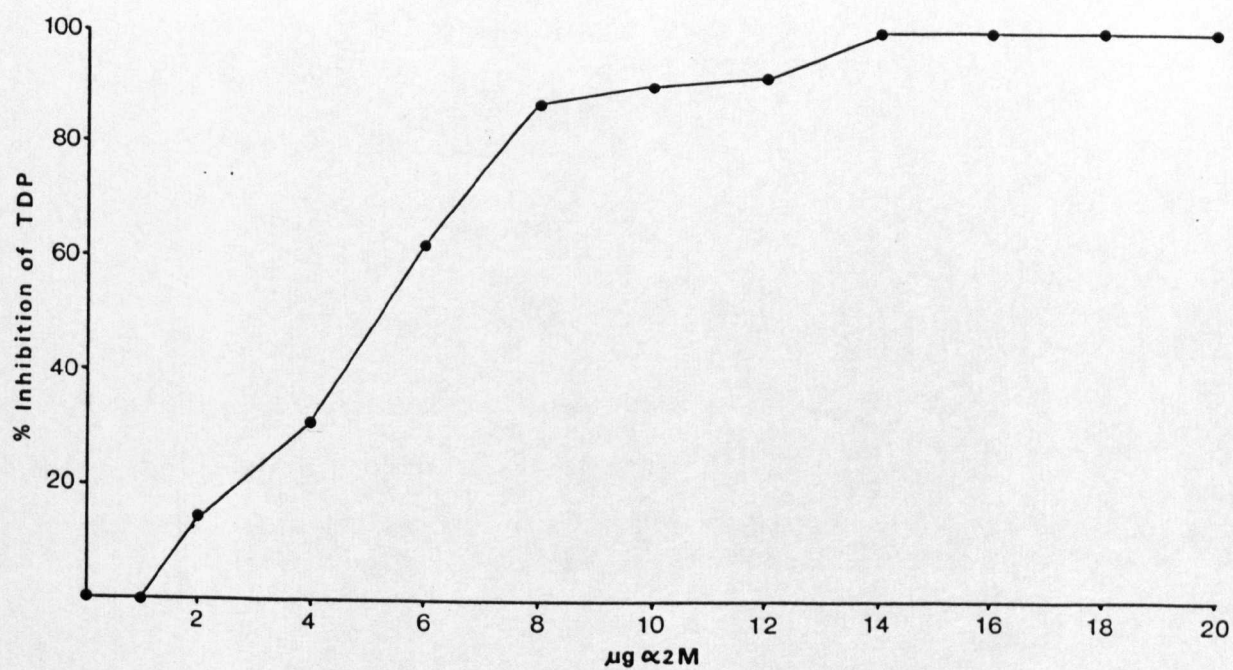


Fig.4.5. Inhibition of TDP activity on azocoll following incubation with various, increasing quantities of $\alpha 2M$. 100% inhibition occurred at a molar ratio of $\alpha 2M$:TDP of 1 : 1.3.

inhibitor, an enzyme:inhibitor molar ratio of approximately 1:1.3. TDP activity was partially inhibited by as little as 2 μ g of α 2M.

4.3.5. REGENERATION OF TDP ACTIVITY

Fig. 4.6 shows the percentage TDP activity remaining after incubation for various lengths of time from 0 to 28 hours with a quantity of α 2M estimated to give 10% residual TDP activity. Two sets of data are shown one of which demonstrates that after 28 hours incubation TDP activity did not significantly increase.

4.3.6. DEGRADATION OF α 2M BY TDP

The effect of TDP on the ability of α 2M to inhibit thermolysin following incubation for various lengths of time is shown in Fig.4.7. When incubated with buffer for 4 hours at 37 $^{\circ}$ C α 2M inhibited thermolysin activity by 100% but when incubated with TDP for the same length of time the thermolysin inhibitory capacity of α 2M was reduced to approximately 90%. This may be accounted for by the occupation of binding sites by the TDP which would block the subsequent binding (and therefore inhibition) of thermolysin. Fig.4.8 shows the results of SDS-PAGE on the reaction mixtures. Incubation with TDP resulted in a change of the α 2M from a predominantly single band of around 180kDa to several bands lower than 100kDa although some material remained at 180kDa (lane 5). Incubation with thermolysin caused a similar, but more complete, alteration of the α 2M with none of the 180kDa band remaining (lane 6).

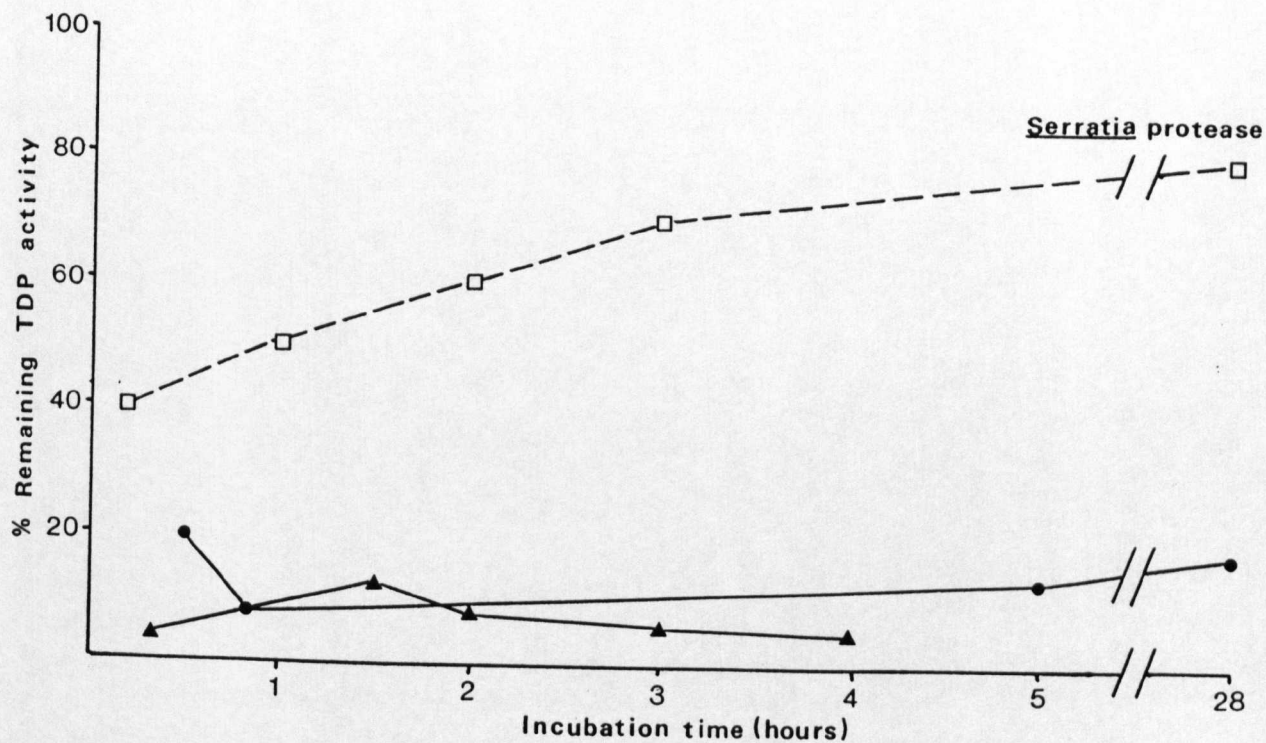


Fig.4.6. Inhibition of TDP activity against Azocoll following prolonged incubation with α 2M at a molar ratio of 1 : 1.8 to look for regeneration of residual activity.

● α 2M+TDP, ▲ α 2M+TDP, □ *Serratia marcescens*
protease (from Molla *et al*, 1986).

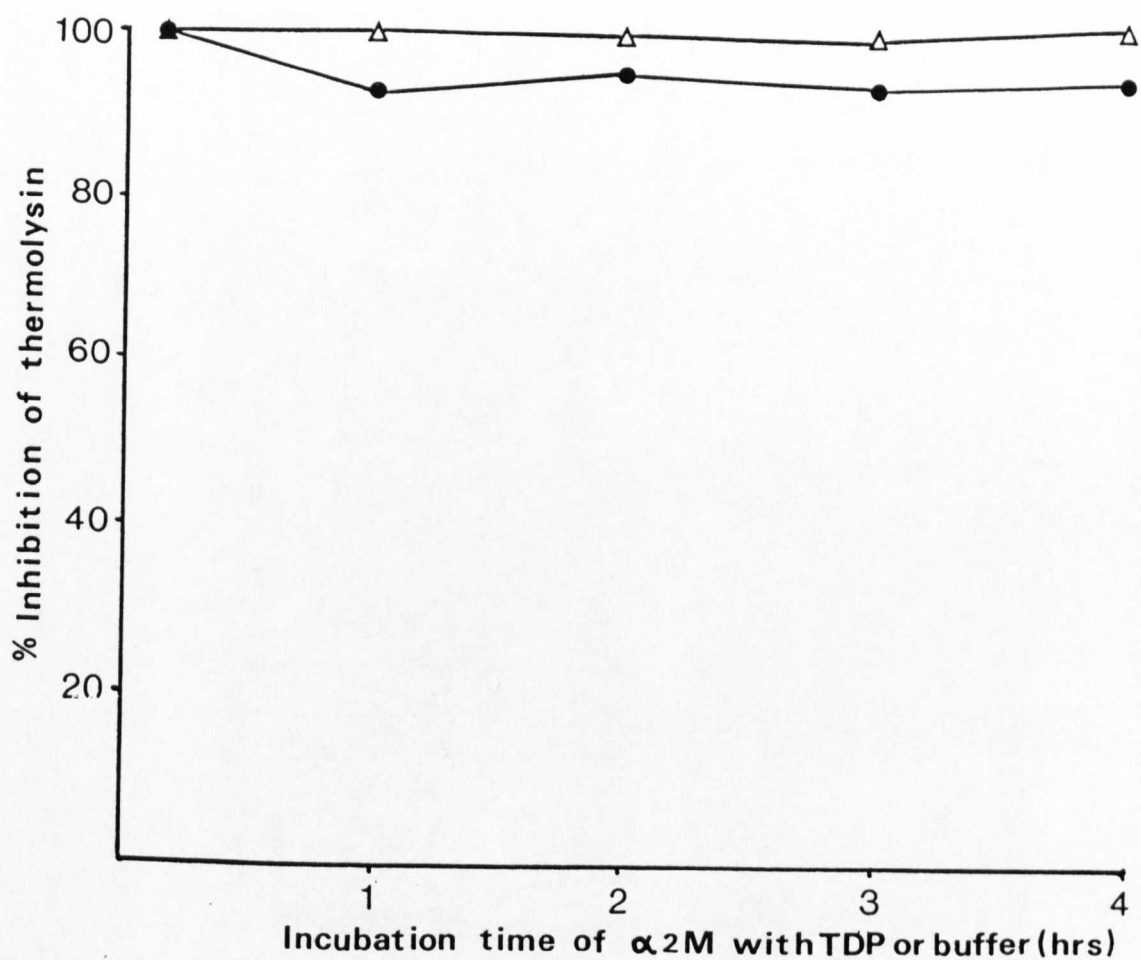


Fig.4.7. Effect of incubation with TDP on the capacity of α 2M to inhibit thermolysin activity against Azocoll.

Δ α 2M + buffer,

\bullet α 2M + TDP

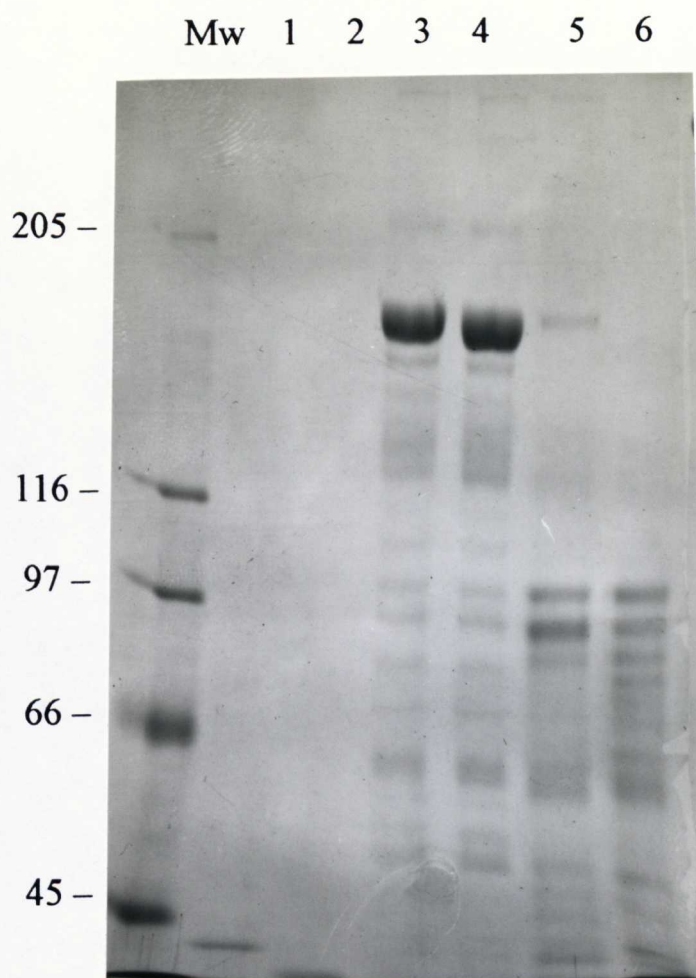


Fig.4.8. SDS-PAGE of reaction mixtures of $\alpha 2M$ incubated with TDP and thermolysin. Lanes:

1. TDP only;
2. thermolysin only;
3. $\alpha 2M$ + TDP 0 hours;
4. $\alpha 2M$ + buffer 4 hours;
5. $\alpha 2M$ + TDP 4 hours;
6. $\alpha 2M$ + thermolysin 4 hours.

4.3.7. PROTECTION STUDIES

Tables 4.1 and 4.2 show temperatures and survival times of guinea pigs challenged with a lethal aerosol of *L.pneumophila* after injection of α 2M or PBS. In experiment A (Table 4.1), four of the control group died on day 2 and all were dead by day 3, an average survival time of 2.2 days, calculated, by dividing the total number of days the animals survived by the total number of animals which died (Fitzgeorge *et al.*, 1986). In the α 2M treated group there was one death on day 2, 4 died on day 3 and one animal survived to day 5, an average survival time of 3.8 days.

In experiment B (Table 4.2), three of the PBS control group had died by day 3 and all were dead by day 4, an average survival time of 3.4 days. The α 2M treated group were all alive on day 3, two died on day 4 and all were dead by day 5, an average survival time of 4.6 days.

Serum levels of α 2M in an unchallenged guinea pig given the same doses of inhibitor as the animals in experiment B (Table 4.2) reached a maximum of 1.4 mg ml^{-1} over the course of the experiment (Fig.4.9). No α 2M was detected in a lung lavage of an unchallenged animal 4 days following similar i.p. injections and these treated animals showed no clinical signs of illness.

4.3.8. INTRACELLULAR PRODUCTION OF TDP

The results of the guinea pig macrophage assay are shown in Table 4.3. Bacterial counts in macrophage lysates increased 100-1000 fold from 0-24 hours. Washing procedures following infection of monolayers resulted in few or no extracellular bacteria at 0 hour but after 24 hours quantities of CV in the supernatants were similar to those in the lysates. Since the culture media did not support the growth of this strain multiplication must have occurred intracellularly.

Table 4.1 Temperatures and survival of guinea pigs treated with $\alpha 2M$ or PBS then challenged with an aerosol of *L.pneumophila*

EXPERIMENT A

Day	Temperature (°C)							Total animals dead (/ group of 5)	
	Control				$\alpha 2M$			Control	$\alpha 2M$
	mean	range	number of animals	mean	range	number of animals			
0	39.2	38.9-39.5	5	39.2	38.9-39.5	5		0	0
1									
24h	40.1	39.8-40.6	5	39.5	39.4-39.8	5		0	0
30h	39.9	39.3-40.3	5	39.9	39.2-40.6	5		0	0
2									
48h	37.6	36.4-39.2	3	39.1	37.5-40.7	5		2	0
55h	35.1	35.1	1	38.6	36.1-40.4	3		4	1
3	-			39.3	39.3	1		5	4
4	-			38.8	38.8	1		5	4
5	-					-		5	5
A.S.T. (days)								2.2	3.8

Table 4.2. Temperatures and survival of guinea pigs treated with $\alpha 2M$ or PBS then challenged with an aerosol of *L.pneumophila*

A.S.T.= Average survival time (see text for calculation).

EXPERIMENT B

	Temperature (°C)						Total animals dead (/ group of 5)	
	Control			α2M			Control	α2M
Day	mean	range	number of animals	mean	range	number of animals		
0	38.9	38.9-39.1	5	38.8	38.7-39.0	5	0	0
1	39.2	39.1-39.3	5	38.9	38.8-39.0	5	0	0
2	39.8	39.3-40.0	5	40.7	39.3-42.0	5	0	0
3	38.3	38.0-38.7	2	38.9	38.7-39.0	5	3	0
4	-			38.4	38.5-38.7	3	5	2
5	-			-			5	5
A.S.T. (days)							3.4	4.6

A.S.T.= Average survival time (see text for calculation).

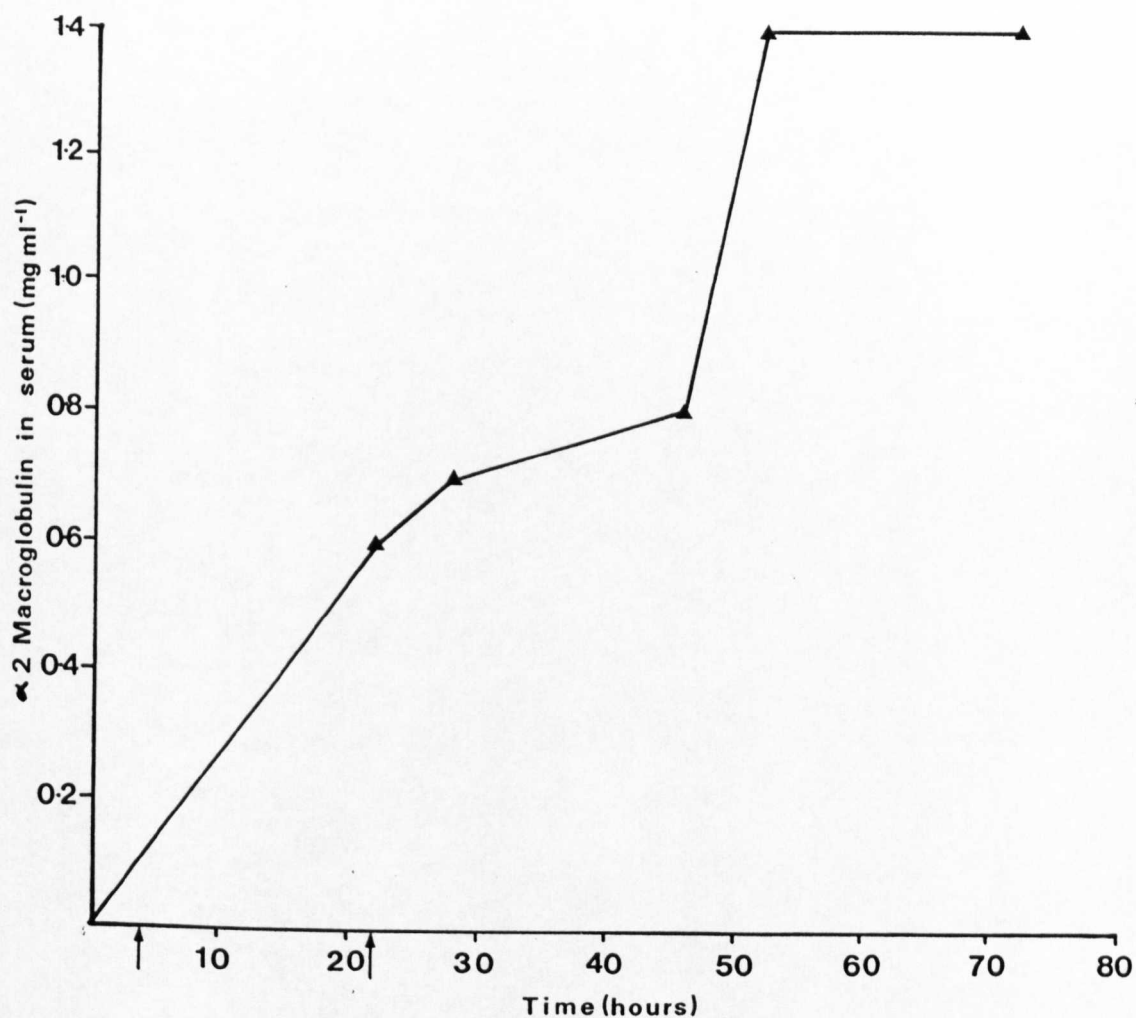


Fig.4.9. Quantities of α_2 M in the serum of a normal guinea pig given intraperitoneal injections of inhibitor in parallel with guinea pigs challenged with *L.pneumophila*. Arrows indicate time of administration at 4 and 24h post infection..

Table 4.3. Production of TDP by *L.pneumophila* in guinea pig macrophages in tissue culture. Values for TDP are the means of duplicate ELISA tests, the range shown in brackets. The results of four experiments are shown.

		SUPERNATANT		INTRACELLULAR	
Time (hours)		viable legionellae c.f.u. / ml	concentration of protease ($\mu\text{g} / 10^6$ c.f.u.)	viable legionellae c.f.u. / ml	concentration of protease ($\mu\text{g} / 10^6$ c.f.u.)
0	a.	0	ND	$2.4-2.5 \times 10^3$	ND
	b.	NT	NT	$2.4-2.7 \times 10^5$	ND
	c.	NT	NT	$2.2-3.6 \times 10^5$	ND
	d.	NT	NT	$4.1-7.1 \times 10^3$	ND
24	a.	NT	NT	NT	NT
	b.	$8.3-9.1 \times 10^6$	0.074 (0.071-0.078)	$5.0-6.9 \times 10^6$	0.221 (0.21-0.232)
	c.	$0.4-8.0 \times 10^7$	0.146 (0.022-0.27)	$1.1-1.6 \times 10^7$	0.106 (0.051-0.162)
	d.	1.5×10^6	0.267	$1.1-2.3 \times 10^6$	0.133 (0.085-0.182)

ND= not detected.

NT= not tested.

Tissue culture medium and lysing agent had no effect on the ELISA and no protease was detected in bacterial suspensions incubated with cell-free culture medium nor at 0 hours. After 24 hours incubation, approximately 0.1-0.2 μ g TDP/10⁶ bacteria was detected in culture supernatants and cell lysates. Table 4.4 shows the comparison of CV with CA and CAC. No multiplication of the avirulent strains occurred between 0 and 24 hours and no TDP was detected by ELISA.

4.3.9. IMMUNOGOLD LABELLING OF TDP

Figure 4.10 a and b show a cell containing numerous bacteria with gold particles marking the presence of TDP which was distributed throughout the phagosome and the cell cytoplasm. Few particles were seen due to the small quantities of enzyme being present in each cell. Fig 4.10c is a cell section incubated with control IgG in place of primary antibody as a negative control and shows no specific labelling.

4.3.10. *IN VIVO* ACTIVITY OF TDP

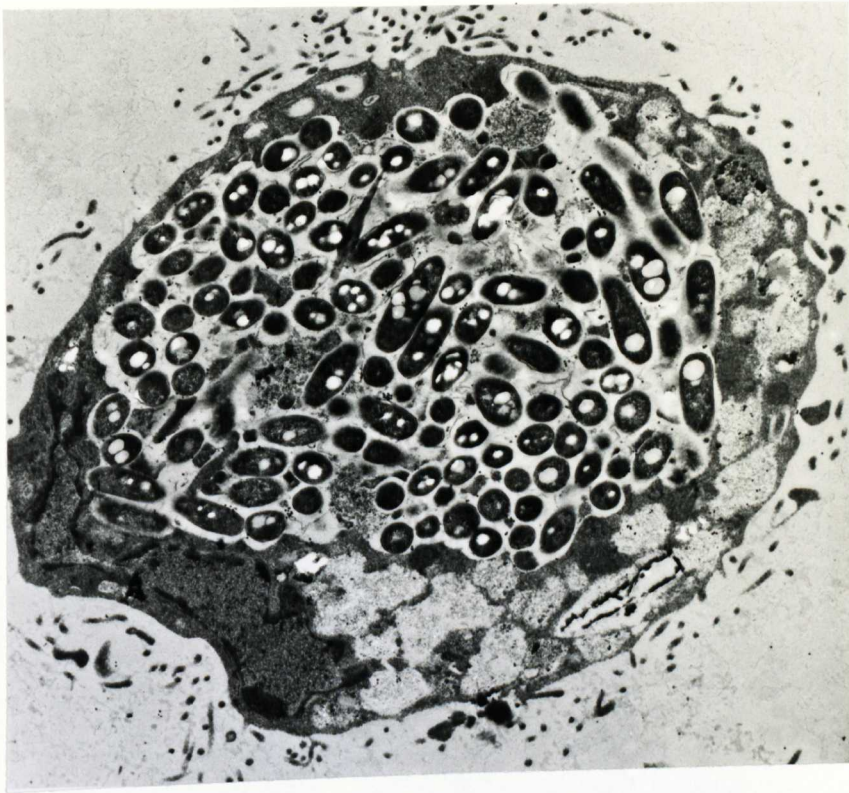
The results of the ELISA for TDP on fractions of infected guinea pig lung lavage lysate which eluted off the Mono Q column are shown in Fig 4.11. The maximum reaction with anti-TDP antibody occurred at a salt concentration of 0.36-0.45M. which was the same as those fractions which caused casein precipitation when broth culture supernatant was separated under equivalent conditions (indicated by shaded area). The TDP-containing fractions were concentrated and applied to a casein agar plate. The resulting precipitation is shown in Fig.4.12., well 1. No precipitation of casein by the elution buffer was seen (well 2) and purified TDP showed strong precipitation (well 3).

Table 4.4. Intracellular production of TDP by Corby virulent and avirulent strains in guinea pig macrophages incubated for 24 hours.

	0 hours	24 hours		
	Intracellular bacteria (c.f.u./ml)	Intracellular bacteria (c.f.u./ml)	TDP by ELISA (µg)	TDP/10 ⁶ c.f.u (µg)
CV	2.4-2.7 x 10 ⁵	5.0-6.9 x 10 ⁶	1.33	0.221
CA	1.3-2.9 x 10 ⁵	5.0-6.1 x 10 ⁵	ND	-
CAC	2.8-3.0 x 10 ⁴	5.8-6.6 x 10 ³	ND	-

ND= not detected

A



B

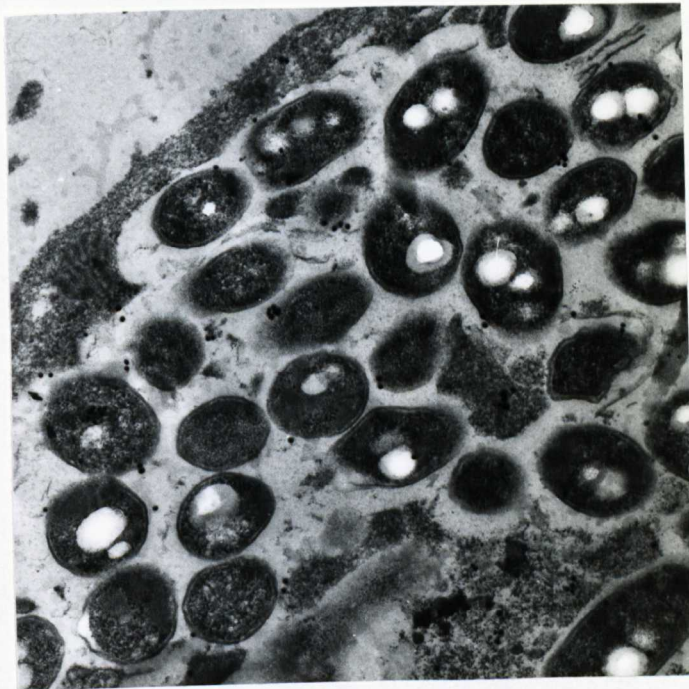
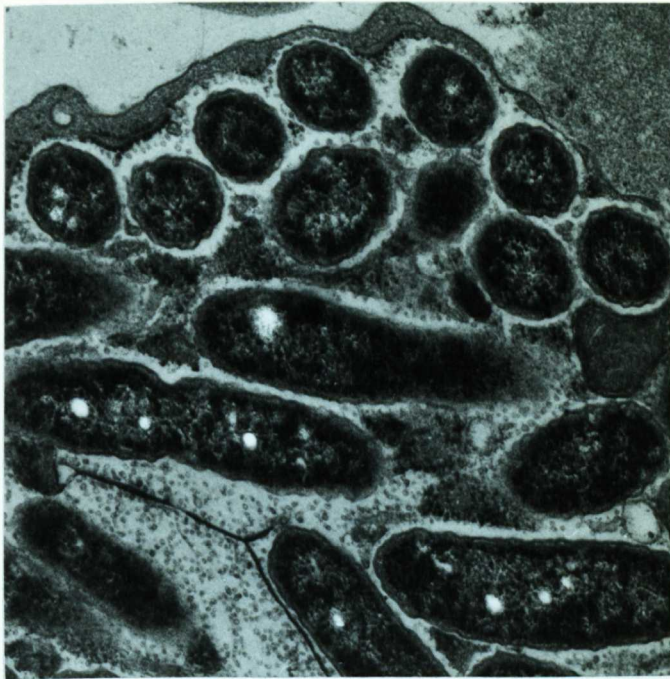


Fig.4.10 . Immunogold labelling for TDP in guinea pig alveolar macrophages infected *in vitro* with *L.pneumophila*.

A, 100 nm section of a macrophage incubated with affinity-purified anti-TDP IgG (x9,600)

B, as A but magnification x 29,000.

C



C, macrophage section incubated with control IgG (x 29,000).

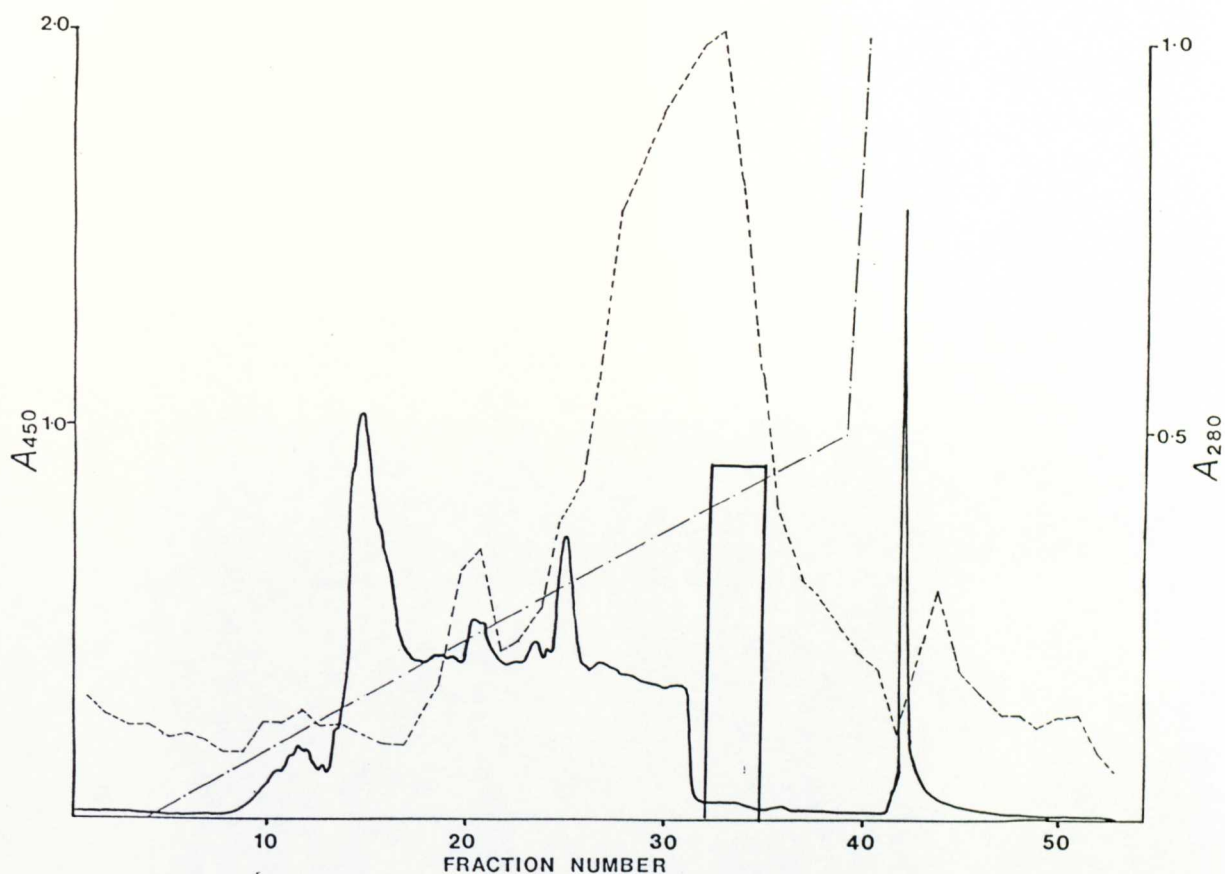


Fig.4.11. Anion exchange chromatography of infected guinea pig lung lavage supernatant using FPLC. Elution of proteins by a salt gradient is shown by absorbance at 280nm. Fractions were assayed for TDP by ELISA and the absorbance at 450nm due to the substrate colour reaction is shown.

— A280; ----- A450; ——— NaCl gradient.

□ - Fractions of broth culture supernatant which caused greatest precipitation of casein following separation under equivalent conditions.

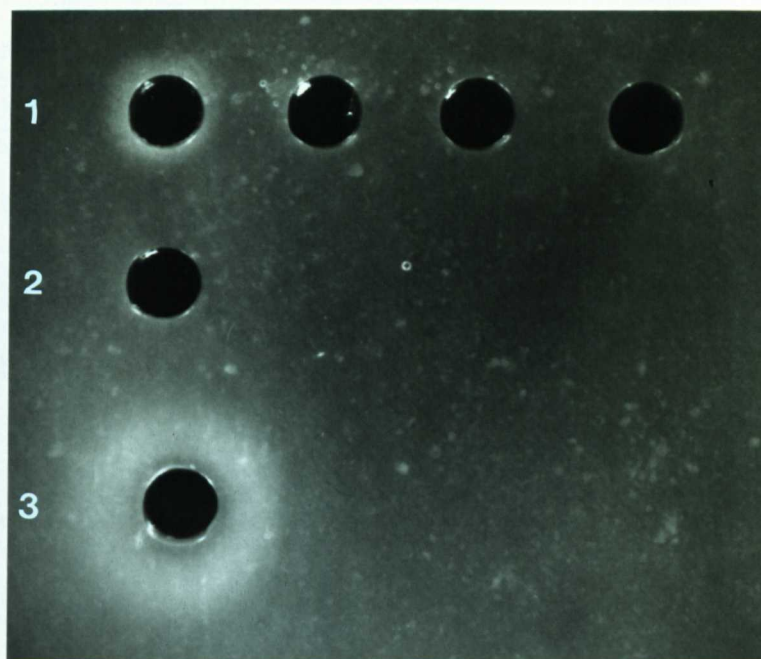


Fig.4.12. Casein precipitation assay of infected lung lavage fractions which gave a positive reaction in the TDP ELISA.

Row 1, doubling dilutions of pooled, concentrated ELISA +ve fractions;

Well 2, buffer control;

Well 3, purified TDP (55 μ g/ml)

4.3.11. COMPARISON OF PROTEASES FROM STRAINS 2029 AND CORBY

Culture supernatant from Philadelphia 1 strain 2029 precipitated casein (Fig. 4.13, well 4) and reacted in the ELISA (data not shown). The zone of precipitation caused by strain 2029 was larger than that produced by CV but, as the numbers of viable bacteria in the broth cultures were not estimated, production of TDP by these strains was not compared quantitatively. Concentrated culture supernatant of the mutant strain 2102 also precipitated casein though to a much lesser degree (Fig.4.13, well 3) indicating that the mutant produced TDP in broth culture.

4.3.12. INTRACELLULAR MULTIPLICATION OF STRAINS 2102 AND 2029

Strains 2102 and 2029 multiplied within guinea pig macrophages *in vitro* at the same rate as each other and a similar rate to CV (Fig.4.14).

4.3.13. VIRULENCE OF STRAINS 2102 AND 2029

Table 4.5 shows the results of challenging guinea pigs with aerosols of 2029 and 2102 in comparison with CV. The LD₅₀ of 2029 and 2102 were similar to each other but much greater than that for CV. Guinea pigs given the parent and mutant strains survived longer than Corby infected animals even though the latter received a much lower challenge dose. There was no significant difference between the parent and mutant strain in the LD₅₀ experiment.



Fig.4.13. Casein precipitation assay of broth culture supernatants of *L.pneumophila* 2029 (parent) and 2102 (mutant) strains in comparison with Corby.

- 1, Broth control;
- 2, Unconcentrated supernatant of 2102;
- 3, Supernatant of 2102 concentrated x100 showing faint precipitation;
- 4, Supernatant of 2029 concentrated x100;
- 5, Supernatant of Corby concentrated x100;
- 6, Purified Corby TDP.

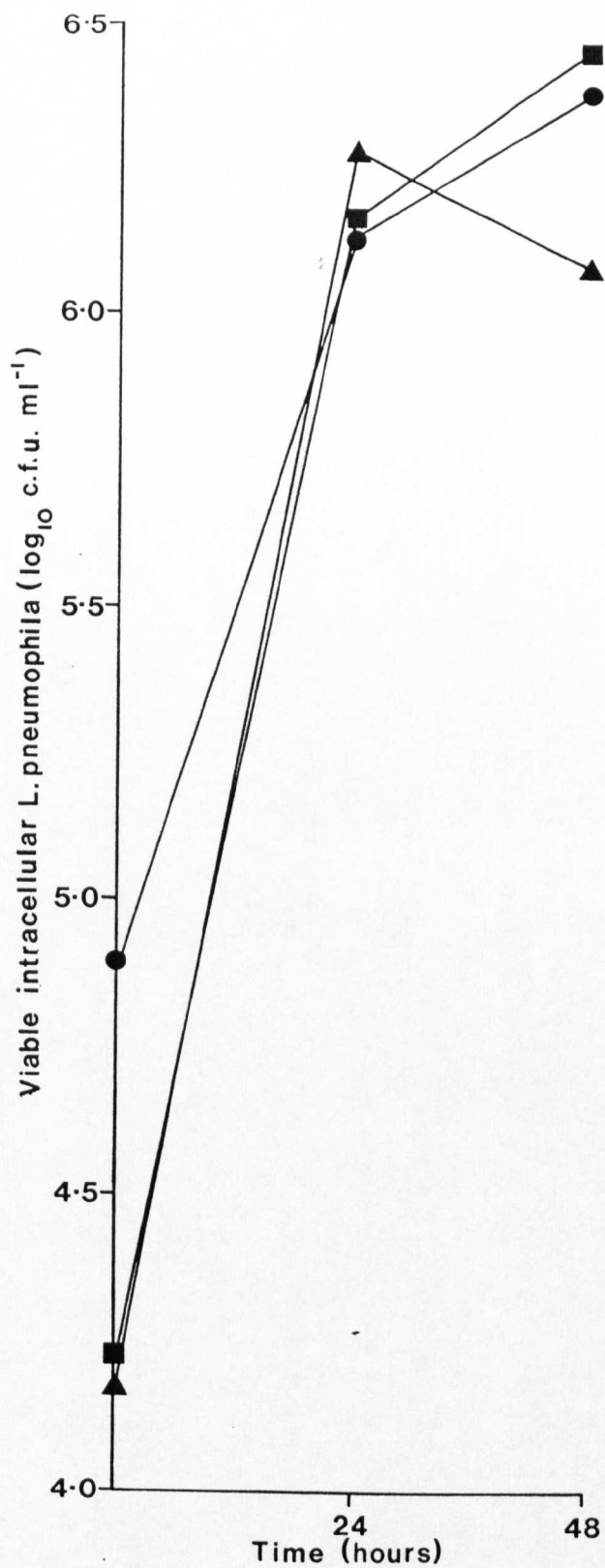


Fig.4.14. Intracellular multiplication of strains 2029, 2102 and Corby within guinea pig alveolar macrophages in cell culture.

▲ - 2029, ● - 2102, ■ - Corby

Table 4.5. LD₅₀ of 2029 and 2102 for guinea pigs infected by the aerosol route and survival rates of guinea pigs following challenge in comparison with CV.

	LD ₅₀	Average survival time (days)
2029	10 ^{5.3}	4.5
2102	10 ^{5.6}	4.7
CV	10 ^{2.3}	3.0

Bacterial counts and TDP production are shown in Figs.4.15. and 4.16. Data for CV is that Conlan *et al* (1988). Multiplication of CV occurred rapidly increasing from 5.7 to 9.3 log₁₀ colony forming units / lung in 2 days. The parent (2029) and (2102) mutant strain multiplied at a much slower rate taking 5 days to achieve similar counts (Fig.4.15). No protease was detected in any of the lung macerates from 2102 infected guinea pigs. Protease was detected after 2 days infection with 2029 and levels increased at a similar though much delayed rate than that produced by CV (Fig 4.16). Protease production by 2029 and CV was at a maximum at the time of death.

Histopathological examination of sections of lung infected with 2029 and 2102 revealed similar pathology though the lesions were fewer and less extensive in the mutant infected lungs than the parent (Fig.4.17)

4.3.14. EFFECT OF CULTURE SUPERNATANT ON GUINEA PIGS

The lungs of guinea pigs treated with concentrated broth culture supernatants of parent and mutant strains are shown in Fig.4.18. The parent strain supernatant induced visible haemorrhage (4.18a) which was

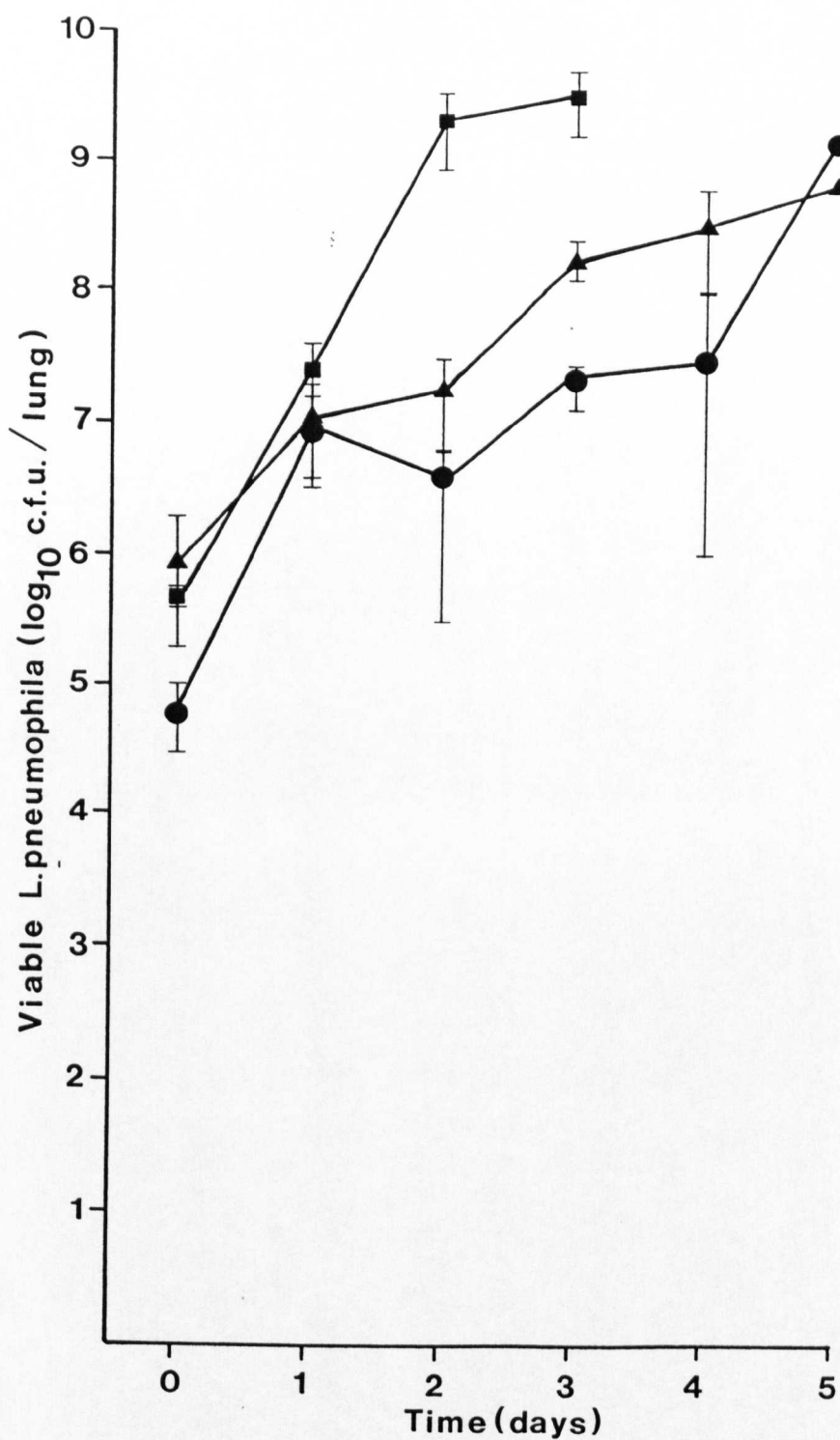


Fig.4.15. Viable *L.pneumophila* in, macerated lungs of guinea pigs infected with strains 2029 and 2102 in comparison with Corby.

■ - CV, ▲ - 2029, ● - 2102.

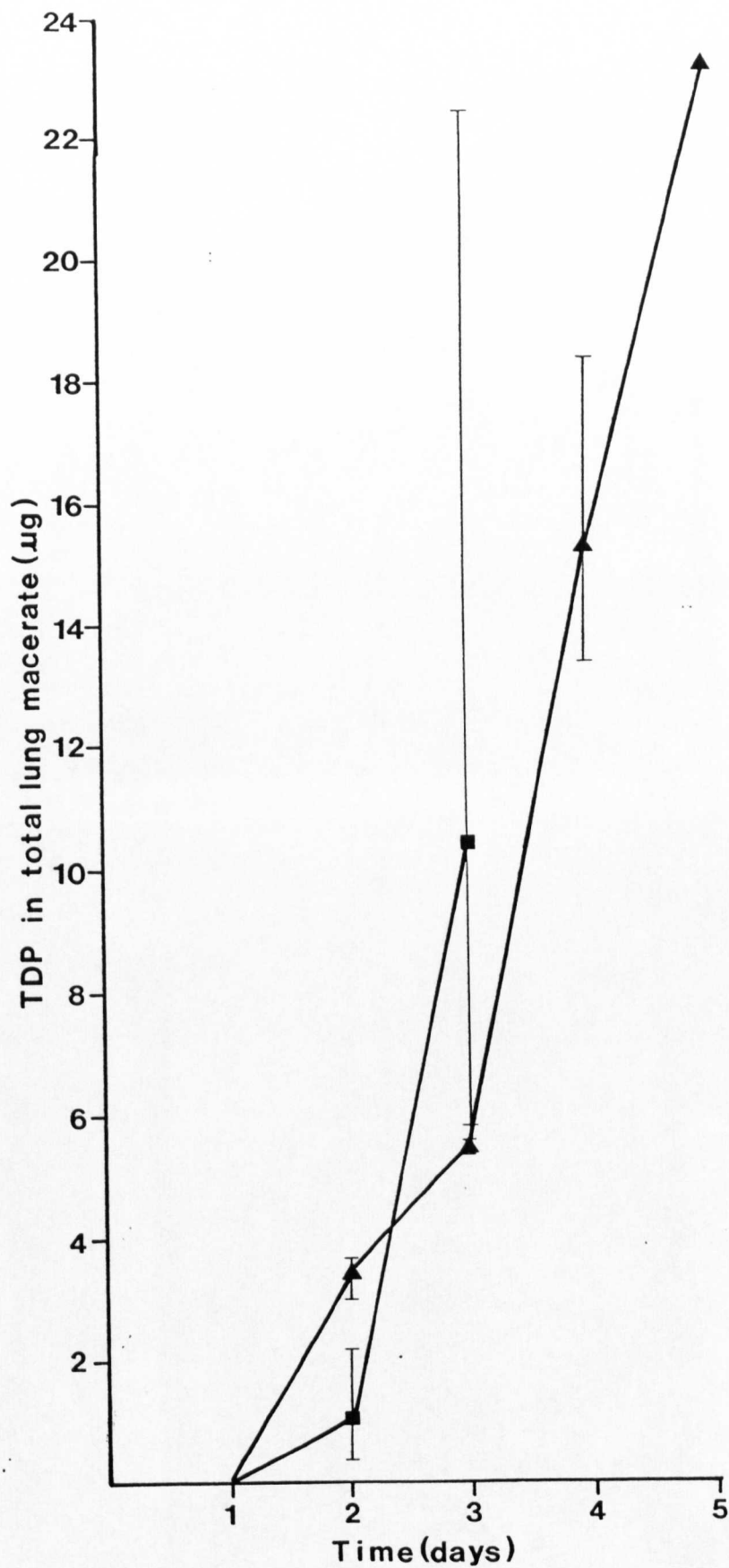
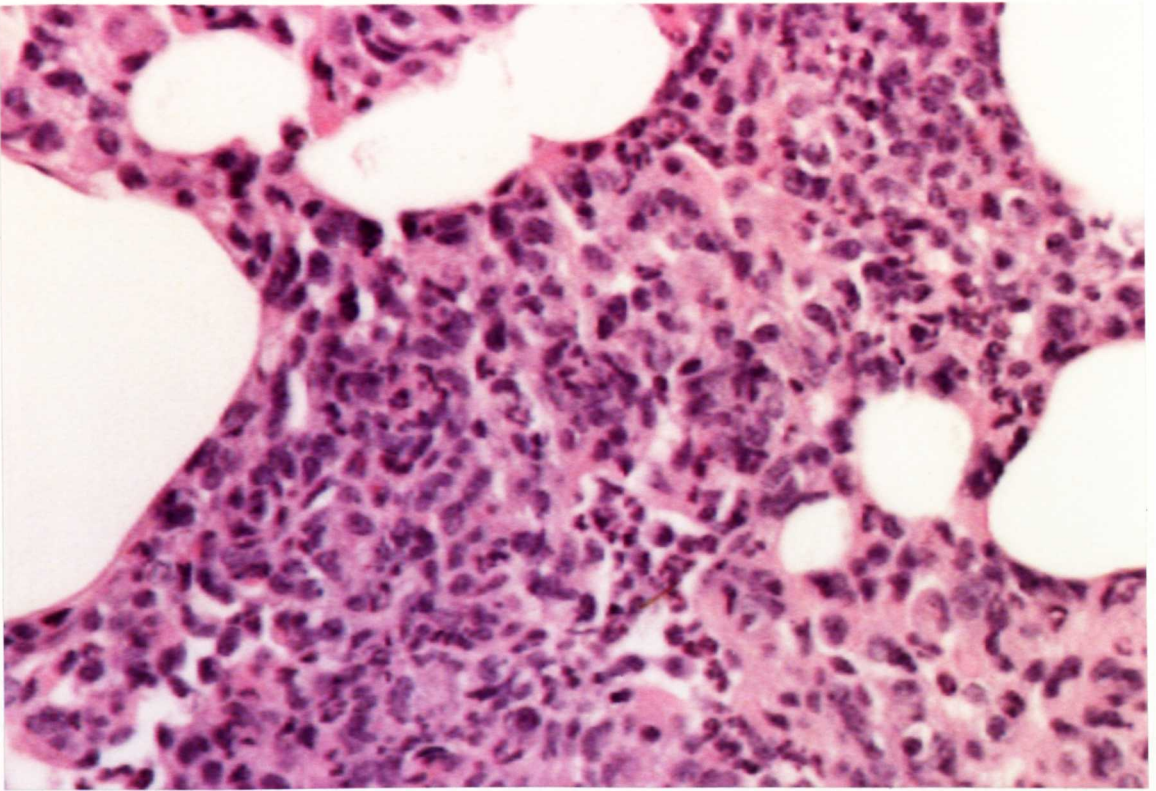


Fig.4.16. Quantities of TDP in macerated lungs of guinea pigs infected with strains 2029 or Corby. ■ -CV, ▲ - 2029.

A



B

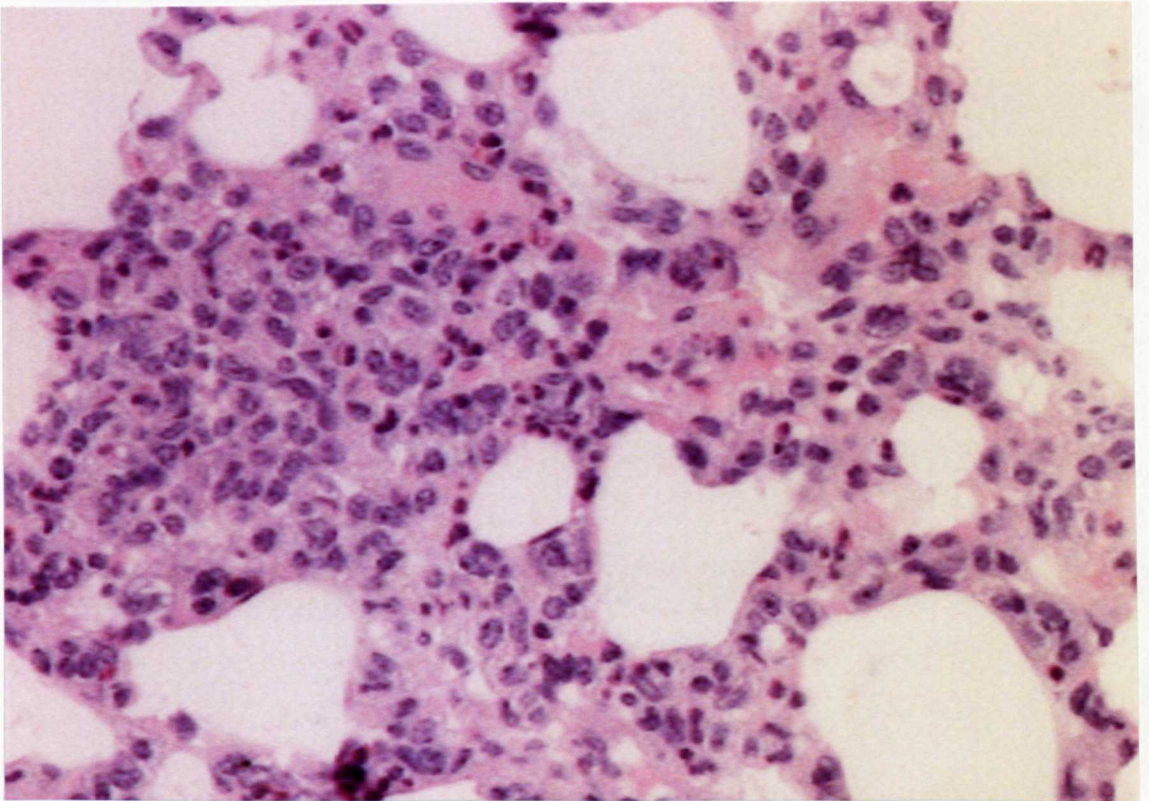
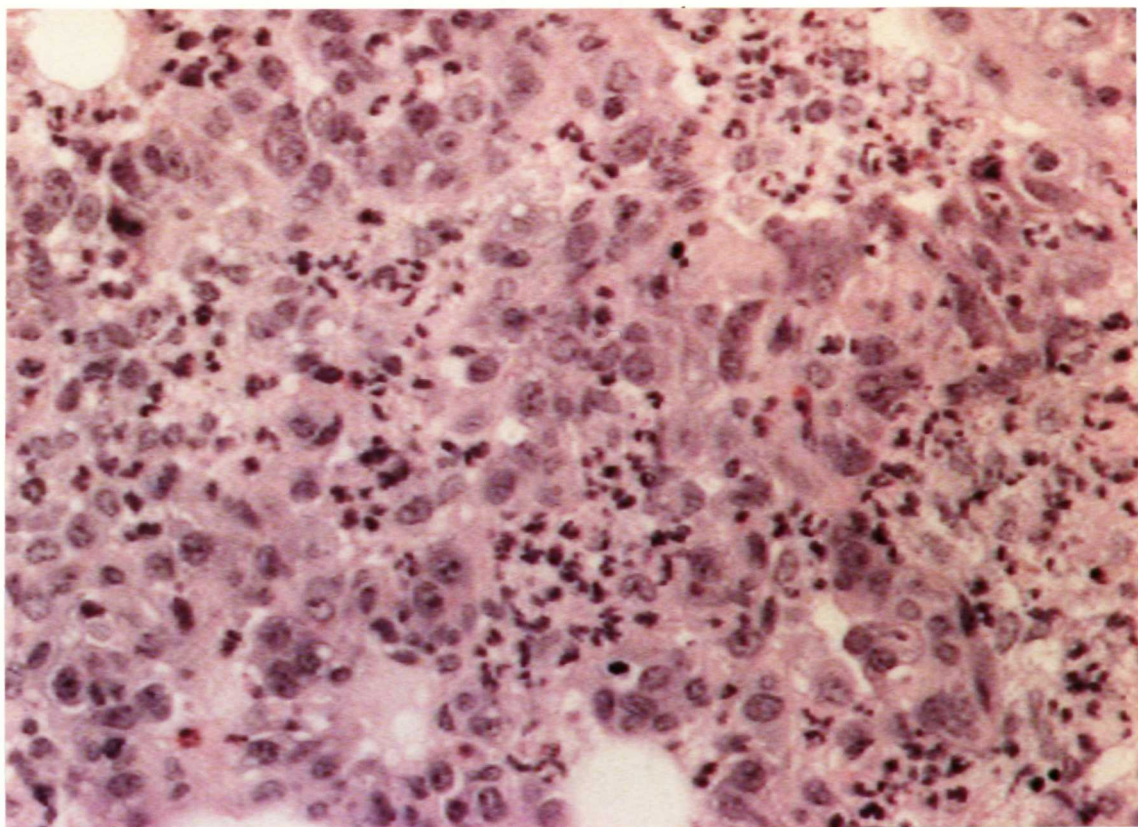


Fig.4.17. Haemotoxylin and eosin (H+E) stained sections of guinea pig lungs following infection with strains 2029 and 2102. Magnification x500.

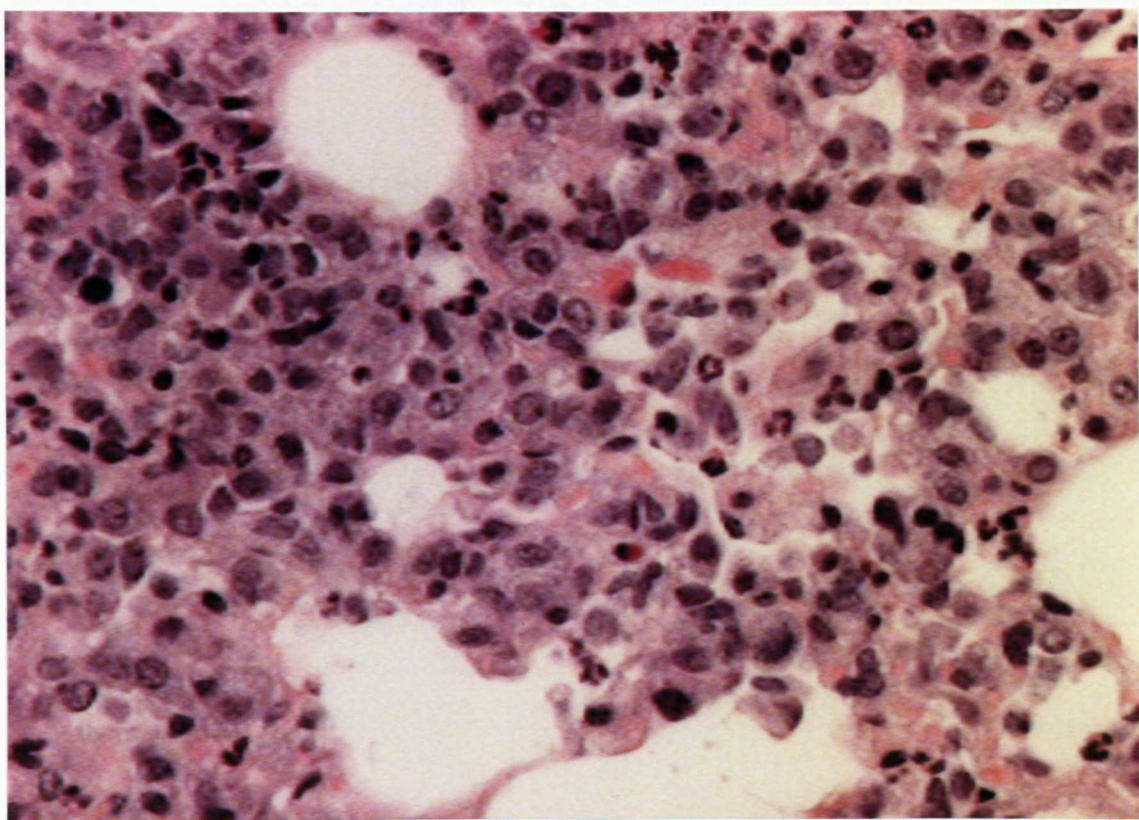
A. 2 days post infection with 2029;

B. 2 days post infection with 2102;

C



D

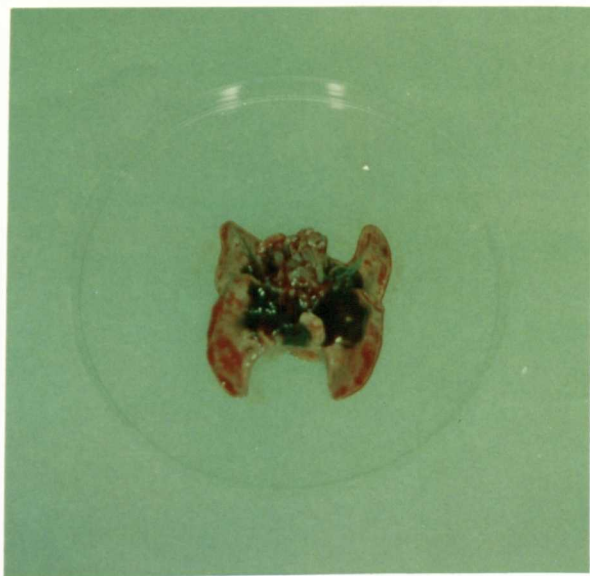


C. 4 days post infection with 2029;

D. 4 days post infection with 2102.

In each case lesions were less extensive in the 2102 infected lungs than 2029 infected lungs

A



B

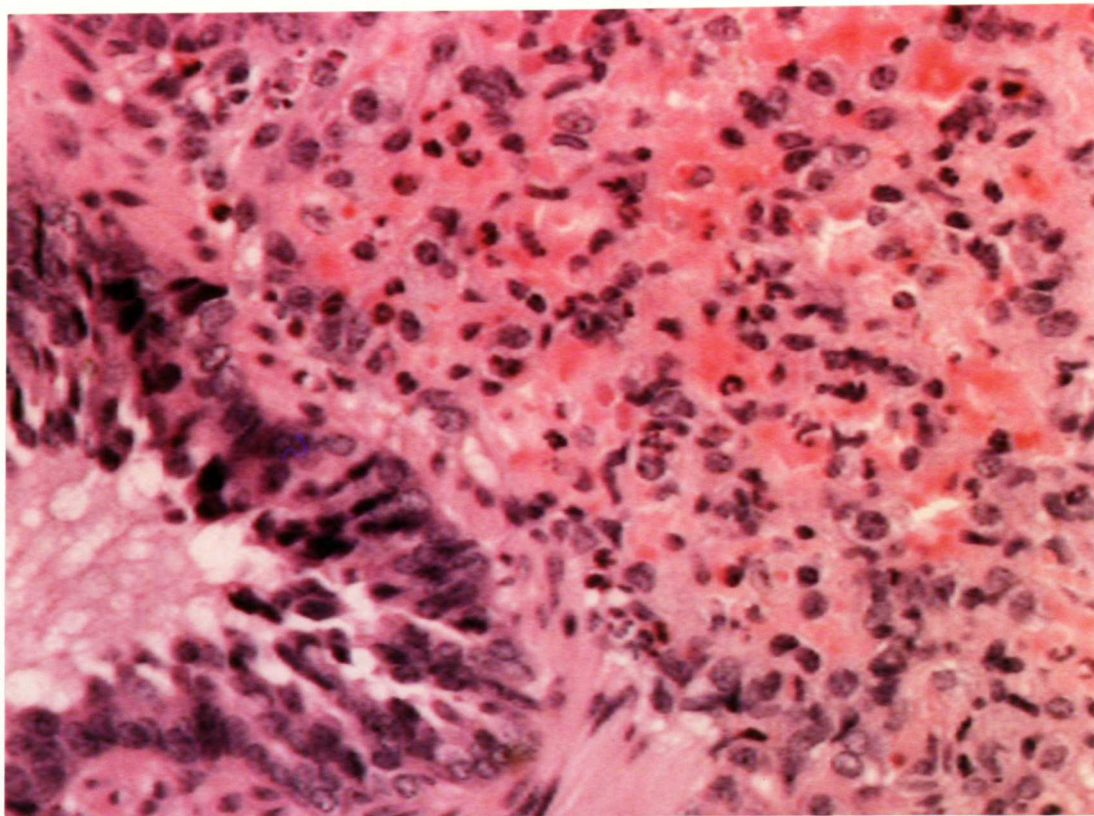


Fig.18. Effects of concentrated culture supernatants of strains 2029 and 2102 when instilled intranasally into guinea pigs.

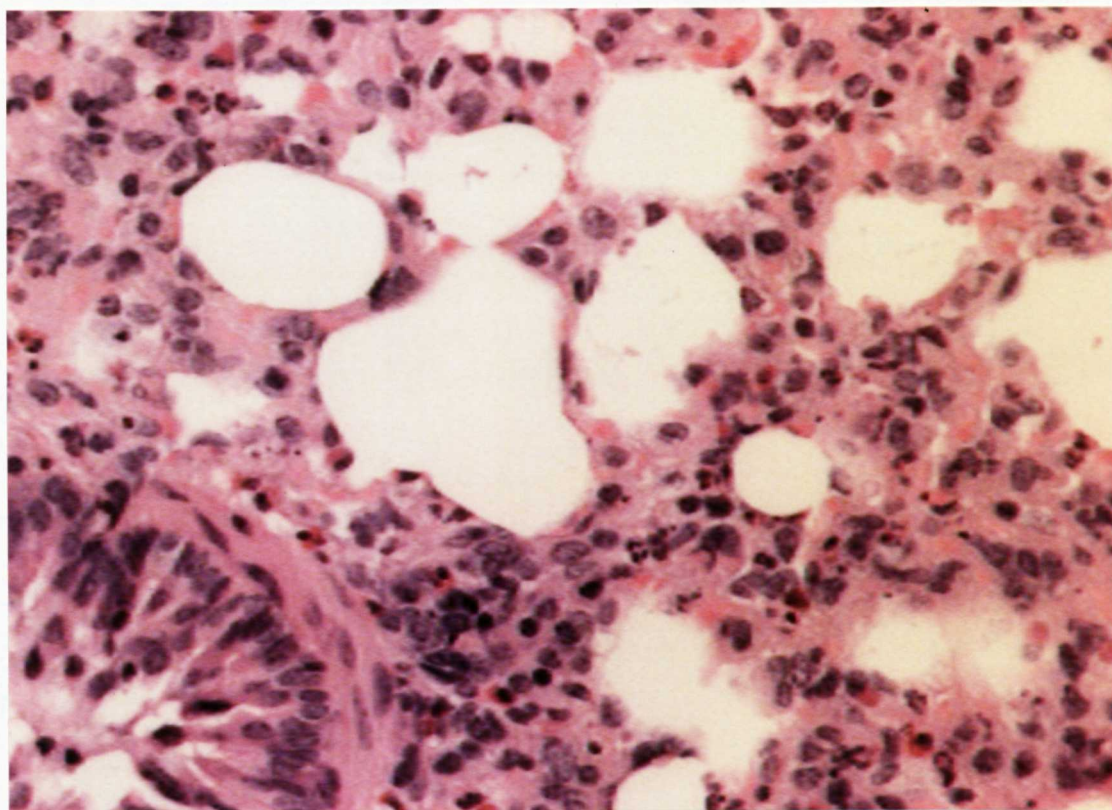
A. 2029 treated whole lung;

B. 2102 treated whole lung;

C



D



C, section of lung treated with 2029, H+E stain, x500;

D, section of lung treated with 2102, H+E stain, x500.

Lungs given 2029 supernatants were more severely damaged than 2012 treated lungs both macroscopically and on examination of tissue sections.

confirmed by histopathology as an acute inflammatory reaction involving extensive tissue damage and cellular infiltration (4.18c). The mutant strain concentrate did not appear to damage the lungs externally (4.18b) though an inflammatory reaction was found to have occurred on examination of lung tissue sections (4.18d).

4.4. DISCUSSION

The pathology of infection by *L.pneumophila* is well characterised as an acute bronchopneumonia involving alveolar tissue oedema which consists of a protein rich fluid containing fibrin and numerous polymorphonuclear leukocytes (PMN) and macrophages. The mechanism by which these lesions are formed has been attributed to the tissue destructive protease which produces identical lesions when administered in a purified form to guinea pigs. Many of the activities of the purified enzyme which have been described in *in vitro* studies also support the hypothesis that TDP is responsible for the lung damage. The experiments reported in this study explore and substantiate a role for TDP in LD pathogenesis.

The *in vitro* experiments in this investigation showed that quantities of TDP, equivalent to those detected *in vivo* in aerosol infected guinea pigs, degraded or inactivated proteins of possible significance to the host in protection against infection with *L.pneumophila*. Gamma interferon (IFN γ) has been shown to inhibit replication of *L.pneumophila* in alveolar macrophages *in vitro* (Nash *et al.*, 1988). Concentrations of human recombinant IFN γ well above those which caused inhibition in the latter study were completely degraded by TDP as shown by SDS-PAGE in this study. Natural IFN γ did not appear to be degraded though this may be due to "quenching" of the protease by serum albumin in which the IFN γ was provided. In a guinea pig intraperitoneal infection model of LD, T-cell proliferation was linked to production of a macrophage activation factor which inhibited intracellular proliferation and had the properties of IFN γ (Nikaido *et al*, 1989). In the guinea pig model of LD used in these studies, virulent strains of *L.pneumophila* multiply rapidly and unabated throughout the course of disease, suggesting that newly recruited macrophages are as

susceptible to the bacteria as those in the initial stages of disease. If TDP does inactivate IFN γ *in vivo*, up-regulation of macrophages could be inhibited, providing a means by which TDP facilitates further proliferation of bacteria intracellularly.

Degradation of Interleukin2 (IL2) by TDP was not clearly shown in these SDS-PAGE studies and only a minor protein band was affected. *Pseudomonas* proteases inhibit proliferation of lymphocytes through cleavage of IL2 which cannot then bind to receptors on the lymphocytes (Theander *et al.*, 1988). It is possible that even a minor alteration in the configuration of the IL2 molecule would interfere with receptor binding but this would have to be proved with an assay of IL2 function following incubation with TDP. Cleavage of IL2 by TDP could interfere with macrophage activation by preventing IL2-mediated T-cell proliferation.

Degradation of immunoglobulins G and A was not apparent by SDS-PAGE studies, though inhibition of specific binding of purified IgG following incubation with the enzyme was demonstrated. IgA binding was not affected, though the saturation of TDP by other proteins in the sample tested cannot be ruled out. The role of immunoglobulins in protection against LD is regarded as being insignificant compared with that of cellular immunity. A humoral antibody response does occur, directed largely at the serogroup antigen but vaccination-induced antibody does not protect against experimental infection with *L.pneumophila* (Baskerville *et al.*, 1983b) The bacteria are resistant to serum killing in the presence of antibody and complement (Horwitz & Silverstein, 1981) but the role, if any, of IgA in the lungs has yet to be investigated. A large number and variety of proteins are found in lungs and, since the relative affinities of TDP for any of these substrates is unknown, *in vitro* observations made with purified substances should be treated with caution.

The importance of a microbial pathogenic or virulence factor can be demonstrated by showing moderation of the effects caused by that factor by an inhibitor. In this investigation TDP was completely inhibited by the protease inhibitor alpha 2 macroglobulin ($\alpha 2M$) *in vitro*. Protease activity was abolished at enzyme:inhibitor ratios similar to those measured for other proteases inhibited by $\alpha 2M$ (Molla *et al.*, 1986). The inhibition appeared to be irreversible with no regeneration of TDP activity on prolonged incubation such as that seen with *Serratia marcescens* protease (Molla *et al.*, 1986, Maeda *et al.*, 1987). Incubation with TDP for up to 4 hours reduced the inhibitory capacity of $\alpha 2M$ but this could be accounted for by the occupancy of binding sites on the inhibitor by TDP. Feldman *et al.*, (1985) have shown that $\alpha 2M$ has two binding sites, therefore after incubation of $\alpha 2M$ and TDP at a molar ratio of 1:0.9 a reduced number of binding sites were available on the inhibitor. Thus the decrease in inhibitory capacity of $\alpha 2M$ shown in Fig.4.7 was not due to degradation by TDP.

There is a discrepancy between these findings and those of Muller (1983) who described degradation of $\alpha 2M$ by *L.pneumophila* strains grown in the presence of serum. Since that study was not on purified protease it is possible that the activity was due to another protease. However, Muller used changes in electrophoretic mobility as an indication of degradation. Barrett (1979) described an increase in electrophoretic mobility of $\alpha 2M$ following binding of proteinases (or amines) due to a conformational change from the "slow" to "fast" form of the molecule. Similar changes in the protein bands on SDS-PAGE gels to those described by Barrett were seen in this study following incubation with TDP or thermolysin (Fig.4.8).

Protection against the lethal effects of *Pseudomonas aeruginosa* proteases by $\alpha 2M$ has been shown in a burned mouse model to be due to inhibition of one of the bacterium's' enzymes (Holder and Haidaris, 1979). There was some evidence in this study that $\alpha 2M$ protected guinea pigs from

infection when the animals were treated with the inhibitor after challenge with a lethal aerosol of *L.pneumophila* (Table 4.1). Although treatment did not prevent death the animals' survival was prolonged by 1.6 days (experiment A) and 1.2 days (experiment B). Since α 2M has a molecular weight greater than 700kDa it is possibly not surprising that no α 2M was found in a lung washout of a treated, uninfected animal. However as a result of the vascular damage seen in this experimental LD model (Baskerville et al., 1983), more α 2M may have been present in the treated, infected animals. It is also possible that levels of α 2M in the lungs were not sufficient to inhibit all the TDP produced. Administration of α 2M by aerosol inhalation rather than intraperitoneal injection may result in greater protection. The increased survival of α 2M treated guinea pigs is unlikely to be due to protection against damage by PMN enzymes since it has been demonstrated that little if any contribution to the lung damage in LD is made by these enzymes (Fitzgeorge *et al* ,1988). The host response to invading organisms in the lung is a complex array of non-specifically induced substances which include proteases and their inhibitors. The effects of introducing a non-specific protease inhibitor such as α 2M are difficult to predict in such an environment since inhibition of host as well as bacterial proteases may occur.

However, given the evidence that TDP has the potential to mediate many of the pathogenic effects of LD it is possible that the prolonged survival was due to a moderation of these effects by α 2M.

The intracellular production of TDP within guinea pig alveolar macrophages was confirmed in this study by ELISA and immunogold labelling of infected macrophages. Quantities produced in the macrophages were less than those detected in guinea pig lungs but given the difference in viable count between infected lungs and this assay (i.e. 100 fold greater in lungs) the amount of TDP produced in the assay could be extrapolated to that found in whole macerated lung. Macrophages are

considered to be the principal site of replication of *L.pneumophila* in the lungs so it is likely that local concentrations of TDP are even higher.

TDP was not produced by avirulent forms of the Corby strain within guinea pig macrophages. Conlan (1987) found that CAC produced TDP when grown in YEB and this study (Chapter 2) showed that, when growth in the culture medium was comparable, there were no differences between the virulent and avirulent pairs in the production of TDP nor any other enzyme activities. However, *in vivo* where the avirulent strains did not multiply, production of the tissue destructive protease either did not occur or was below the detection limits of the ELISA. Thus the avirulent strains have the ability to produce TDP but, *in vivo*, multiplication is required in order to produce sufficient quantities of the enzyme. Since some of the activities of TDP involve inhibition of anti-microbial strategies (Rechnitzer & Kharazmi, 1992, Sahney *et al.*, 1993) it is possible that, without TDP, avirulent strains could be cleared from the lungs more readily than virulent ones.

The use of an immunological assay does not confirm that the enzyme detected *in vivo* is active. In this study functional activity of TDP purified from lungs of guinea pigs with experimental LD was demonstrated by caseinase activity of material which reacted in the ELISA for TDP. Thus it was shown that *L.pneumophila* produces large quantities of a highly active and tissue destructive enzyme *in vivo* which is supportive of an important role for TDP in the pathogenesis of LD.

Comparisons of the protease (MSP) produced by the Philadelphia 1 strain, which was used to prepare a protease-minus mutant, with that produced by CV, demonstrated that the two enzymes were immunologically and biochemically similar. When given to guinea pigs as a fine particle aerosol both parent and mutant bacteria had very low virulence. In order to cause disease, very large doses of the organism had to be administered and the guinea pigs took several days to die (Table 4.4).

However parent and mutant forms had similar LD₅₀ values and TDP was detected *in vivo* by ELISA of lung macerates from animals infected with the parent strain but not in those infected with the mutant. This confirmed that production of protease was not required for death of guinea pigs. There was a distinct difference in the progression of disease caused by the Philadelphia strains compared to Corby, suggesting an inherent difference in the pathogenesis of the three strains. Histopathological evidence has indicated that the degree of severity of alveolar necrosis varies considerably in different outbreaks and is characteristic of the disease-causing strain of *L.pneumophila* (Baskerville, 1988). Where such variations occur any one pathogenic / virulence factor may be more or less critical in causing morbidity or mortality. In the case of the parent/mutant strains alternative disease-causing factors could be another enzyme or a toxin. Examination of the lungs infected by the parent strain, and following administration of culture supernatant, revealed lesions typical of LD in the guinea pig (Baskerville, personal communication and Figs 4.17 a & c, 4.18c) which were either caused by the protease or a substance with similar activity. Such lesions were also found in the lungs of guinea pigs infected with the mutant strain (and in those given culture supernatant) but were less extensive than those caused by the parent strain (Figs 4.17b & d, 4.18d). In their original description of these strains Szeto & Shuman (1989) reported that the mutant exhibited a residual casein precipitating activity approximately 1000 fold less than the parent strain and this was also demonstrated here (Fig.4.13). If low levels (undetectable by ELISA) of protease were produced by the mutant *in vivo* it is possible that the lung lesions were caused by TDP and it cannot be concluded that the strain causes death independently of protease production. Since the levels of protease were below the detection limits of the ELISA it is likely that other factors contributed to the lung damage seen following

infection with the mutant. Given the 'leaky' nature of the mutant strain and the low virulence of it and its parent strain, the evidence based on these strains does not exclude a role for TDP in the pathogenesis of LD.

The enzyme may not be the single factor which determines the outcome of disease in the Philadelphia 1 strain of *L.pneumophila* but the experiments reported in this Chapter have added to the existing evidence of a pathogenic role for TDP by demonstrating more potential host-modulating activities and proving intracellular production and *in vivo* activity. It seems likely that TDP is one of a number of virulence factors which contribute to the pathogenesis of Legionnaires' disease.

CHAPTER 5

An immunocompromised mouse model of Legionnaires' disease

5.1. INTRODUCTION

Investigations of the pathogenesis of most infectious diseases are greatly aided by an animal model which exhibits the same characteristics as the disease in humans. Such a model not only allows elucidation of the pathogenic mechanisms but is vital in evaluation of therapeutic measures to control or eradicate the disease.

A variety of animal systems have been used to assess virulence and pathogenesis of *L.pneumophila* which include embryonated hens eggs (McDade & Shephard, 1979), and various cultured cells such as epithelial cells (Daisy *et al.*, 1981), alveolar macrophages from various animal species (Kishimoto *et al.*, 1979; Fitzgeorge, 1985; Rechnitzer *et al.*, 1992), blood monocytes (Horwitz & Silverstein, 1981) macrophage-like cell lines (Pearlman *et al.*, 1988) and free living protozoa (Rowbotham, 1980; Fields *et al.*, 1986.)

Apart from marmosets, Rhesus and vervet monkeys (Baskerville *et al.*, 1983) the only whole animal model to successfully mimic the disease of humans is the guinea pig. However many of the guinea pig models used involve infection via the peritoneum or trachea which Fitzgeorge *et al.* (1983) showed to be inappropriate routes in determining virulence of *L.pneumophila* strains. The aerosol infection model used throughout the studies in this thesis induces a pneumonia in guinea pigs typical of that seen in humans and has been used extensively to study virulence pathogenesis diagnosis and treatment of LD (Baskerville, 1988). There are, however, limitations to the use of guinea pigs not least of which is that these animals

do not have an immune deficiency, a factor usually predisposing humans to Legionnaires' disease (LD)

The severely compromised immune deficient (SCID) mouse has a genetic defect under the control of a recessive (*scid*) gene which results in deficiency of immune functions mediated by T and B lymphocytes (Bosma *et al.*, 1983). These animals are highly susceptible to opportunistic infections and have been used as models of human diseases which affect immunocompromised individuals (Mead *et al.*, 1991). Since the main risk groups for LD are immune deficient, the SCID mouse may be a suitable model for this disease when an aerosol route of infection is used. The SCID mice used in this study were a variety (beige, bg) bearing not only the SCID mutation but additional defects in natural killer (NK) cells and possibly other granulocytes. Granulocytes have been shown to limit the spread of *L.pneumophila* infections (Fitzgeorge *et al.*, 1988) and NK cells have a possible role in defence (Blanchard *et al.*, 1988) indicating that the SCID/bg mouse would be susceptible to LD. To investigate this, SCID/bg mice were exposed to aerosols of *L.pneumophila* in comparison with Balbc mice which do not have an immune deficiency

SCID mice do not have the ability to reject foreign material and are therefore able to tolerate cells from other species and, since the defect is mainly in lymphocytes, any foreign lymphoid or stem cell introduced can become a part of a normal immune system (Bosma *et al.*, 1983). Introduction of human cells therefore results in a mouse with a partially human immune system. Models of human disease should ideally involve species as closely related to man as possible so disease models in humanised SCID mice have the potential to be extremely useful. The opportunity to use such mice presented itself through experiments which were in progress to humanise SCID/bg mice using peripheral blood leukocytes. The mice were assessed for susceptibility to *L.pneumophila*

aerosol infection with the intention of developing a model to investigate cellular mechanisms operating during infection.

5.2. MATERIALS AND METHODS

5.2.1. SCID/bg MICE.

The SCID/bg mice used in these experiments, described by McDougall *et al.* (1990), were obtained from the University of Guelph, Guelph, Ontario. They were bred in-house under the supervision of Dr. B.W. McBride who kindly provided these mice and those which had been reconstituted with human peripheral blood lymphocytes.

5.2.2. EXPOSURE OF MICE TO AEROSOLS OF *L.PNEUMOPHILA*.

Mice were challenged with a lethal aerosol of *L.pneumophila*, serogroup 1 Corby strain as described in Chapter 1 using the Henderson apparatus connected to a 3-jet collision spray to generate aerosols from a water suspension (1×10^{10} colony forming units ml⁻¹) of the bacteria and delivered to the snouts of the mice. The mice were exposed to the aerosol for exactly 5 minutes after which time 2 mice were killed and their lungs removed for counts of viable legionellae. At daily intervals mice were observed for signs of illness and a proportion were killed and their lungs removed for viable bacterial counts and histopathology. Bacterial viable counts were performed on macerated half portions of lungs which were serially diluted in distilled water, 0.2 ml aliquots of dilutions were plated onto BCYE agar and incubated for 3 days at 37°C. The corresponding half lungs were either fixed in 10% buffered formal saline and processed for wax embedding or deep frozen prior to cryo-embedding and sectioning. Tissue sections 5µm thick were stained with haematoxylin and eosin and by immunocytochemical labelling with antibodies to *L.pneumophila* LPS (see Chapter 2) and human lymphocyte surface markers. Histopathological analysis was performed by Dr. G.A. Hall and immunocytochemical analysis by Dr. B.W. McBride.

5.2.3. INTRACELLULAR FATE OF *L.PNEUMOPHILA* IN SCID/BG ALVEOLAR MACROPHAGES

The lungs of 8 SCID/bg mice were washed out with tissue culture medium (MEM containing 5U Heparin ml⁻¹), 4 hours after aerosol challenge with *L.pneumophila*. Viable bacterial counts were performed on the lavage fluid before and after the cells were removed by centrifugation at 200g for 10 min. A portion of the cell pellet was removed and the cells disrupted by adding 200 µl 0.8% (w/v) digitonin and incubating for 10 minutes at 37°C. The cell lysate was diluted in distilled water and plated onto BCYE to determine the number of viable, intracellular *L.pneumophila*. The remainder of the cells were resuspended in MEM containing 5% newborn calf serum and were counted in a Neubauer haemocytometer. The volume was adjusted to give a concentration of 1×10^5 - 10^6 macrophages ml⁻¹ and 200µl aliquots added to wells of a 96 well tissue culture plate (Nunc) and allowed to settle at 37°C for 30 min. Non-adherent cells and extracellular bacteria were then washed away. The final wash was plated onto BCYE to check that all bacteria had been removed. Viable bacterial counts were performed on the tissue culture medium and washed, disrupted cells after 48 hours incubation at 37°C.

5.3. RESULTS

5.3.1. CHALLENGE OF SCID/BG MICE WITH CV

Following aerosol administration of 16 SCID/ beige mice with 1×10^{10} viable CV ml⁻¹ no signs of illness were observed in any of the animals and an initial 0 hour bacterial count of around 7×10^5 viable Corby ml⁻¹ was gradually reduced over 9 days (Fig.5.1).

When the SCID/bg and Balbc mice were challenged with CV in parallel neither strain showed signs of illness and the viable counts of CV in the lungs showed a similar reduction with time (Fig.5.2).

5.3.2. INTRACELLULAR FATE OF CV IN SCID/BG MACROPHAGES

Table 5.1 shows that 4 hours following challenge with CV the pooled lung lavages of 8 mice contained 2.5×10^4 c.f.u. ml⁻¹ and the majority of these bacteria were extracellular. The lavage cells when placed in tissue culture contained no recoverable intracellular CV but 6.9×10^2 bacteria ml⁻¹ were present in the culture medium. After 48 hours incubation no viable bacteria were found associated with the cells despite 8×10^3 bacteria / ml being present in the culture medium.

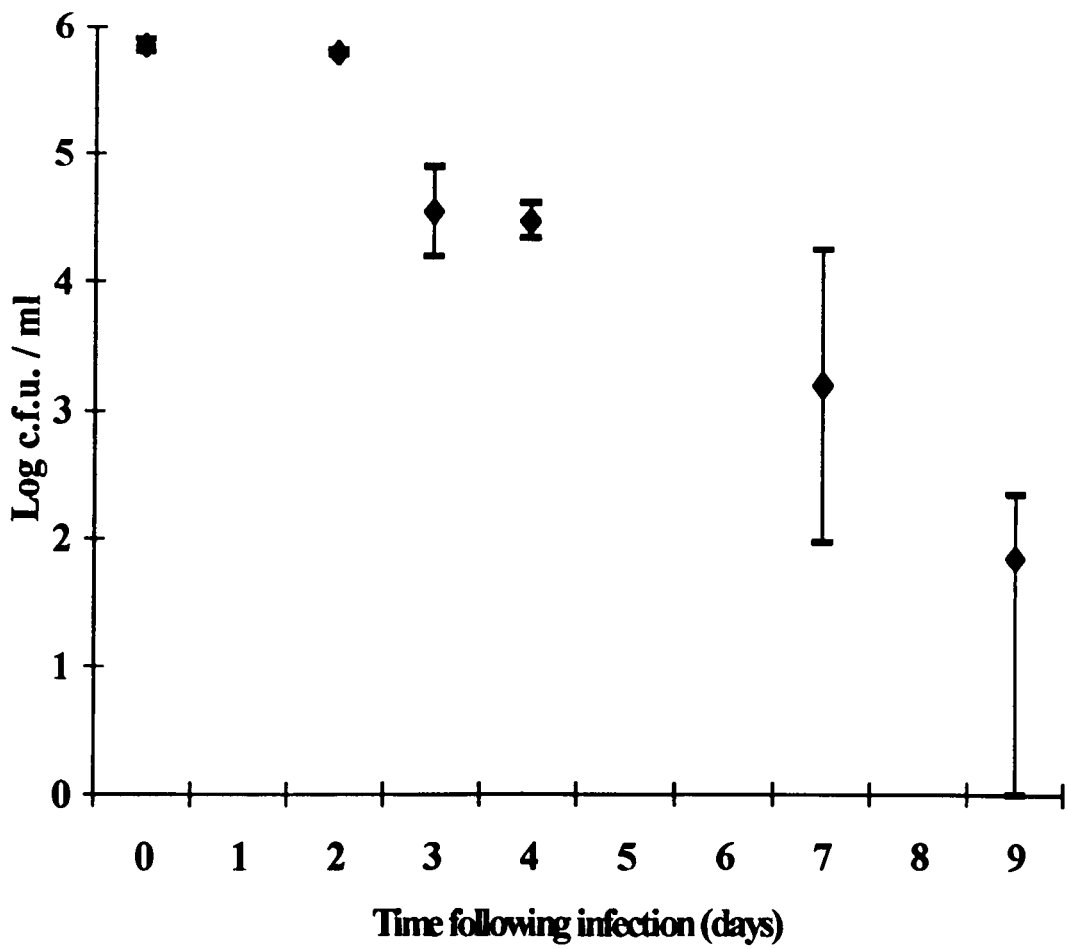


Fig.5.1. Viable, bacterial counts of *L.pneumophila* in the lungs of SCID/beige mice following aerosol challenge.

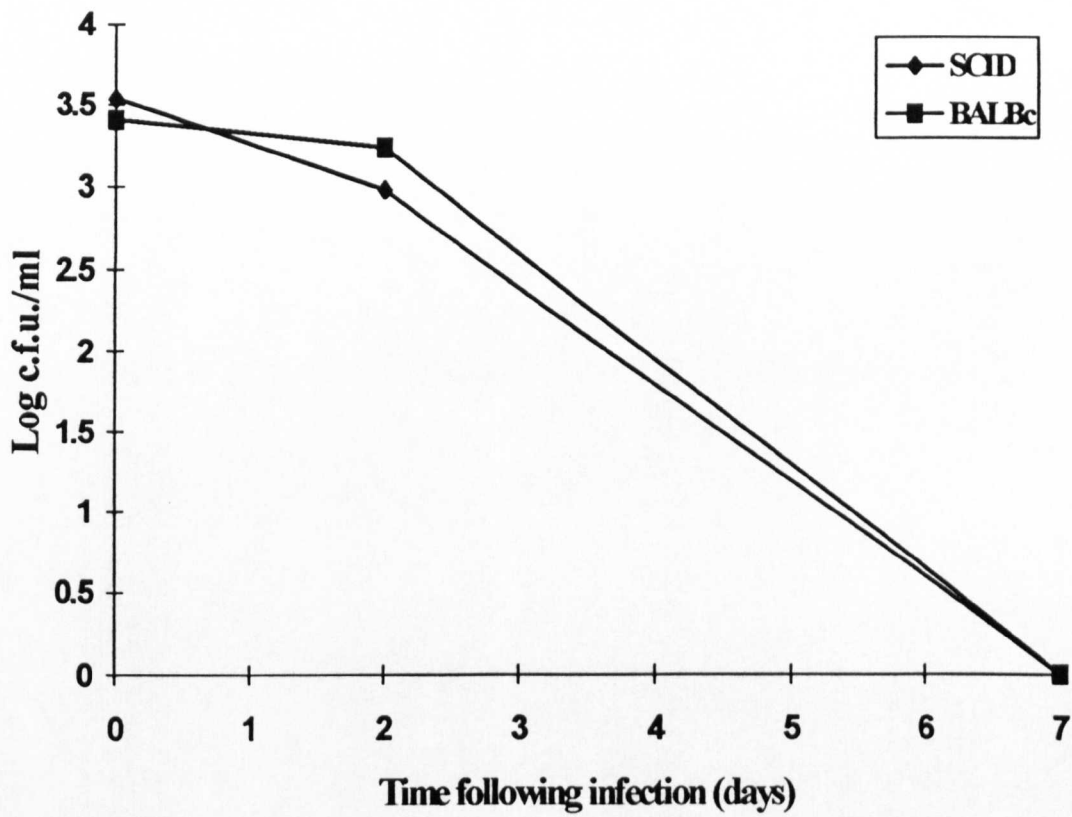


Fig.5.2. Viable, bacterial counts of *L.pneumophila* in the lungs of SCID/beige and Balbc/c mice following aerosol challenge.

Table 5.1. Viable counts of intra and extracellular *L.pneumophila* in SCID/bg mouse lung lavages after challenge with CV and following incubation of cells in tissue culture.

	Viable count (c.f.u./ml)	
whole lavage	2.5 x 10 ⁴	
extracellular fraction	2.27.x 10 ⁴	
	Viable count (c.f.u./ml)	
	0 hours	48 hours
cells in tissue culture	nd	nd
culture supernatant	6.96 x 10 ²	8.05 x 10 ³

nd = not detected.

5.3.3. CHALLENGE OF RECONSTITUTED SCID/BG MICE WITH CV

SCID/bg mice which had been treated with human peripheral lymphocytes were challenged with a potentially lethal aerosol of CV. Viable counts of CV in the lungs are shown in Fig 5.3. Multiplication occurred with viable counts increasing from 10⁵ up to 10¹⁰ colony forming units/ml. These mice also became ill and the arrows indicate animals which died. There were three deaths in total, two on day 1 and one on day 2. Some of the mice did not become ill and in this group the numbers of viable CV decreased with time.

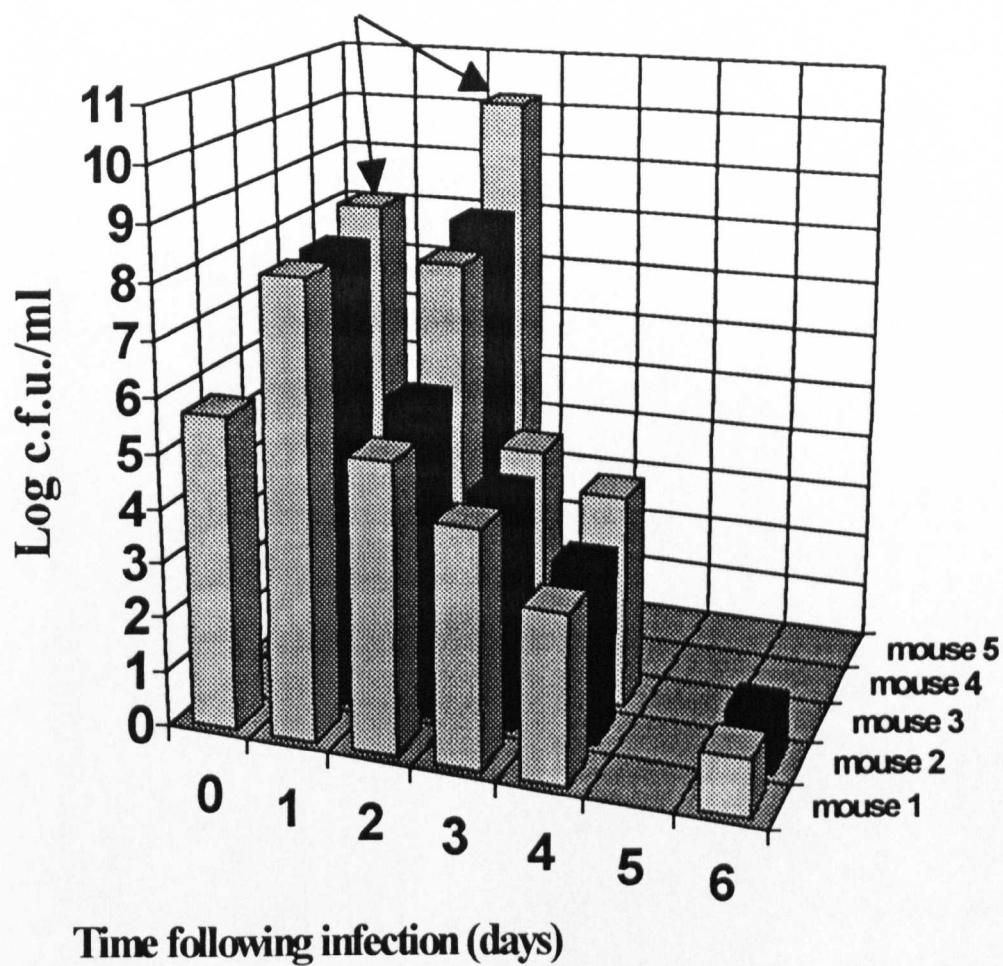


Fig.5.3. Viable *L.pneumophila* in the lungs of reconstituted SCID mice following aerosol challenge. The arrows indicate mice which died. Deaths were at day 1 and day 2.

5.3.4. HISTOLOGY OF LUNGS OF RECONSTITUTED SCID/BG MICE INFECTED WITH CV.

The analysis of infected lung sections stained with haematoxylin and eosin as interpreted by histopathologist Dr.G.A. Hall was as follows. Two distinct types of pathology were seen one of which was characterised by inflammation and tissue damage and the other by thickening of the alveolar walls and the presence of large numbers of macrophages.

Immunostaining of tissue sections with anti-*L.pneumophila* LPS gave a variable reaction which was related to the numbers of legionellae obtained by viable counts. Fig.5.4 shows representative results of this labelling in a section of lung from an animal which had an increase in the numbers of legionellae compared with 0 hours.

Human macrophages and lymphocytes were not observed in tissue sections stained with appropriate antibodies.

Table 5.2. summarises the results of immunostaining and histopathology in relation to viable counts.

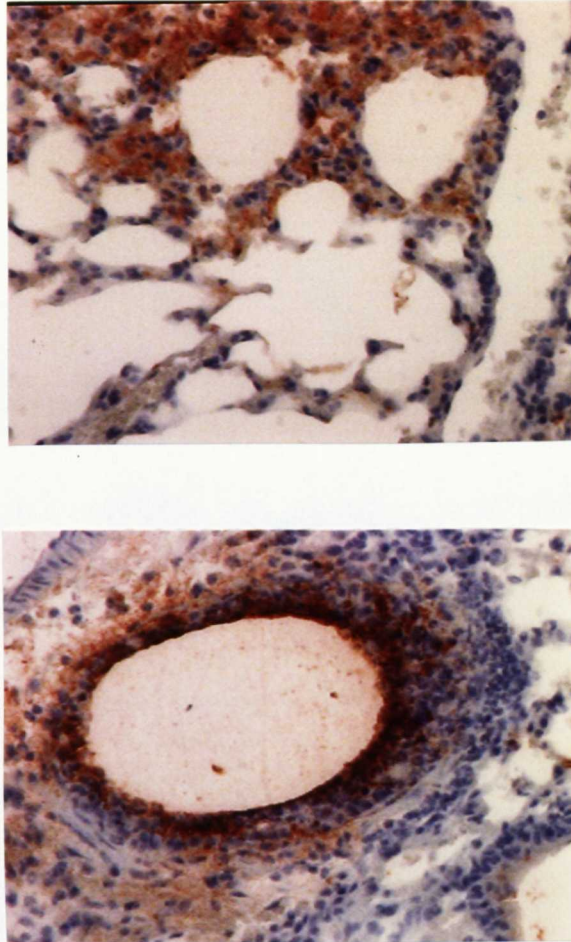


Fig.5.4. Photomicrographs of lungs of SCID/beige mice reconstituted with human peripheral lymphocytes challenged with *L.pneumophila* and immunostained with an antibody against *L.pneumophila* LPS. The bacterial antigen is stained brown and the tissue counterstained with haematoxylin. Magnification, x125.

Table 5.2. Summary of viable counts and histology.

Days post infection	Viable count minus that at 0h (log ₁₀ c.f.u.)	Score of specific labelling for CV	Category according to pathology
1	3.03	nt	nt
1	2.56	+++	A
1	2.52	+++	A
2	4.44	nt	nt
2	-0.49	+/-	C
2	0.03	-	C
2	2.11	+++	A
2	2.5	+++	A
3	-1.48	+	C
3	-1.17	+/-	C
3	1.67	+++	A
3	-1.46	+/-	C
4	-1.83	-	C
4	-2.58	-	C
4*	-2.75	+++	C
6	-4.75	-	C
6	-4.75	-	C

nt = not tested.

Category A = 'Acute' i.e. Inflammation and tissue damage

Category C = 'Chronic' i.e. Thickening of alveoli, macrophages.

* This animal may have had infection in only one lobe of the lung which was processed for histology and not bacteriology.

5.4. DISCUSSION

Cell mediated immune responses are widely reported as being the most important defence mechanisms against invasion by *L.pneumophila* yet the results of the present study found that an animal with a defect in its immune cells was able to resist infection by the aerosol route. Despite having a severe immune deficiency the SCID/bg mice were as able to clear a lethal dose of *L.pneumophila* from the lungs as the Balbc mice. Neither of the mouse strains showed any signs of illness. To investigate the resistance of SCID/bg mice to *L.pneumophila* infection the intra- and extracellular environments of the lungs were examined immediately following challenge. No viable bacteria were recovered intracellularly suggesting that the SCID/bg macrophages either did not phagocytose the legionellae or that they were killed very efficiently upon entry to the cell. Whichever is the case will be determined in future experiments but it is likely that the SCID mice do not succumb to disease following aerosol challenge because the macrophages do not support the growth of *L.pneumophila*.

The *scid* mutation affects differentiation of lymphocytes such that no functional T or B cells mature from stem cells but myeloid cell differentiation is not affected (Dorshkind *et al.*, 1984). Bancroft *et al.*, (1986) have shown that peritoneal macrophages of SCID mice respond to lymphokines in culture, with enhanced expression of Ia molecules. Such activation of macrophages was also observed following infection with *Listeria monocytogenes*. Thus the non-permissiveness of SCID/bg macrophages for legionellae was perhaps not surprising but further demonstrates the requirement for a defect in macrophage defence mechanisms for *L.pneumophila* to establish an infection. In the study mentioned above (Bancroft *et al.*, 1986), SCID mice which were infected with *Listeria* were unable to inhibit the spread of infection and unlike

control mice (without the mutation) developed chronically high loads of bacteria. The apparent discrepancy is due to the fact that T-cell dependent responses are critical in the control of infection by this organism and suggests that this is not the case for *L.pneumophila*. Most strains of mice are not susceptible to LD due to an inability of *L.pneumophila* to multiply within mouse alveolar macrophages (Yamamoto *et al.*, 1987). Peritoneal macrophages obtained from an inbred strain of mice (A/J) allowed growth of *L.pneumophila* (Yamamoto *et al.*, 1988) but the whole mice were resistant to intravenous challenge with the bacteria (Yamamoto *et al.*, 1992) because these mice elicit T-cell responses capable of inhibiting growth of legionellae. The resistance of A/J mice to infection with *L.pneumophila* demonstrates that, when macrophages do allow replication of legionellae, lymphocyte function is important in determining susceptibility or resistance. However, it should be noted that the latter studies involved peritoneal not alveolar macrophages and the route of infection was intravenous not by aerosol. As mentioned previously the route of infection and use of the most appropriate model of the *in vivo* environment can be critical in determining the pathogenesis of disease. It would be interesting to discover the outcome of aerosol infection of A/J mice with *L.pneumophila* and to examine the susceptibility of alveolar macrophages.

The results reported here clearly demonstrated the importance of the non-specific, innate defences of the lung since, without any immune cell involvement, the legionellae were cleared from the lungs as efficiently as mice with intact immune systems.

SCID/bg mice reconstituted with human peripheral leukocytes were found to be susceptible to infection with the Corby strain of *L.pneumophila*, multiplication occurred with viable counts increasing from 10^5 up to 10^{10} colony forming units/ml. These mice also became ill and

death occurred rapidly in some animals. Some of the mice did not become ill and in this group the numbers of viable CV decreased with time.

Examination of the lung tissue by histopathological techniques revealed that the animals in which there was multiplication of CV showed signs of inflammation and tissue damage. Lung tissue of the mice which did not support growth of CV was filled with macrophages causing a thickening of the alveolar walls. Immunostaining for CV correlated to the bacterial lung counts. A hypothesis to explain these different responses is that the lungs of the mice which became ill contained a greater proportion of permissive human macrophages/monocytes than the mice which cleared the infection. The pathology in the lungs of the ill mice is consistent with the release of *L.pneumophila* pathogenic factors and infiltration with inflammatory cells. It is possible that only a few permissive macrophages could initiate the effects seen since *L.pneumophila* exoproducts, in particular TDP, have been shown to deplete host defences in numerous ways (see Chapter 4). The large numbers of macrophages seen in the lungs of resistant mice would have to be non-permissive mouse macrophages for this hypothesis to be correct. Immunostaining with human lymphocyte markers showed that neither human T and B cells nor macrophages were present in the lung sections. This would appear to confirm that the macrophages in the resistant group of mice were of mouse origin. The lack of staining in the mice which allowed growth of CV could be explained by degradation of these cells by the bacteria or even masking of the surface antigens by the large quantities of *L.pneumophila* antigens.

Reconstitution of the mice with peripheral blood lymphocytes resulted in a promising but inconsistent model of LD but perhaps this was not the most effective method to model for an infection the pathogenesis of which is centered around a specialised cell located within the lungs. However *L.pneumophila* challenge of the mice following reconstitution

with isolated populations of human alveolar macrophages in comparison with macrophages plus human T cells could determine the relative contributions of these cell types to defence against LD and provide a consistent, highly relevant model of LD.

CHAPTER 6

Concluding Discussion

6.1. COMPARISON OF VIRULENT AND AVIRULENT *L.PNEUMOPHILA*.

The aims of the studies presented in this thesis were to provide information which contributed to the understanding of the virulence and pathogenesis of *Legionella pneumophila* in an experimental model of Legionnaires' disease (LD). Examination of virulent and paired avirulent strains revealed that an ability to multiply within cultured alveolar macrophages was a characteristic of strains which caused disease in guinea pigs, yet no factor was clearly identified upon which this ability was dependent. Similar findings have been reported (Horwitz, 1987) and it would appear that avirulent forms of *L.pneumophila* which have been produced by passage on artificial media do not exhibit changes in any one of the putative virulence factors described to date. The reason for this could be either that the factor which the avirulent strains lack has yet to be discovered or, that several factors are required for virulence of *L.pneumophila* and a combination of these are defective in avirulent strains.

In these studies, avirulent *L.pneumophila* were phagocytosed by guinea pig alveolar macrophages, so it is events which occur after this process which discriminated virulent and avirulent bacteria. Defective triggering of the oxidative burst of macrophages by virulent legionellae compared with paired avirulent forms has been reported (Summersgill *et al.*, 1990) as a reason why virulent and not avirulent strains are able to multiply intracellularly. A candidate for the inhibition is the mip protein described in Chapter 2 which has protein kinase C (PKC) inhibitory activity (Hurley *et al.*, 1993). PKC is believed to be involved in the

initiation of the oxidative burst by signalling the reduction of oxygen to superoxide anion by NADPH oxidase (Clark, 1990). Expression of mip by both CA and CAC was shown in the present studies but, as neither the quantity nor activity of the protein were determined, it was not possible to state whether the mip was functional. However, in the macrophage assay, CA and CAC did not display the same pattern of intracellular viability which has been described for mutants lacking mip (Cianciotto *et al.*, 1989b) i.e. inhibition in the early stages, followed by multiplication, indicating that the avirulent forms had not lost mip activity. The involvement of mip in the early stages of intracellular replication was thus not clearly demonstrated in these experiments. A possible reason is that the mip protein does not exert the proposed effect on guinea pig alveolar macrophages since the suggested role of PKC in the early stages of the respiratory burst is based on evidence derived from experiments on PMNLs. Alternatively, another bacterial factor could be involved which has a more significant effect on the anti-microbial activation of macrophages than the action of mip. A low molecular weight toxin, produced by *L.pneumophila* which, when incubated with PMNLs, inhibits their subsequent oxidative response to a phagocytosed particle by preventing membrane depolarization (Lochner *et al.*, 1985) could be such a factor. The requirement for pre-incubation of PMNLs with the toxin suggests that it would not be involved in the initial encounter of CV with macrophages but, as there is no information regarding the effects of the toxin on macrophages, nor has it been reported whether avirulent strains produce the toxin in equivalent quantities to virulent strains, the possible involvement in the early intracellular events is speculative.

A role for TDP in the disruption of phagocyte defences has been mentioned in Chapter 4 but, like the toxin, the effects reported required

pre-incubation of PMNLs and monocytes with the enzyme (Rechnitzer & Kharazmi, 1992).

At present the mip is the only reported protein which seems likely to have an effect in the early stages of the intracellular survival of *L.pneumophila* but its mode of action has yet to be clearly defined and, given that mutants lacking mip are still able to multiply intracellularly and that the avirulent strains in the present study expressed the protein it is probable that other factors are involved. Whatever the cause, the inability to repress the oxidative burst of macrophages is a plausible reason why avirulent strains do not multiply within these cells. However the evidence for this theory is based on experiments performed on monocytes and PMNLs *in vitro* and, although there were significant differences between each virulent and avirulent pair of strains, variations between strains were such that some avirulent bacteria were more inhibitory than virulent forms (Summersgill *et al.*, 1988 & 1990). It was also shown that, while both virulent and avirulent legionellae had the ability to inhibit the oxidative burst, the activation of these cells was not prevented and some toxic oxygen species were produced. The fact that virulent bacteria are able to multiply therefore implies a resistance to these metabolites. Differences in the susceptibilities of virulent and avirulent *L.pneumophila* to the xanthine-xanthine-oxidase system (the end products of which are the same as that produced during the oxidative burst), in particular to hydrogen peroxide and to a lesser extent superoxide anion, have been shown (Jepras & Fitzgeorge, 1985). These studies were performed on CV and CAC and it was found that CV was more resistant to hydrogen peroxide and superoxide anion than CAC. This resistance was probably due to a greater production of catalase and superoxide dismutase (respectively) by CV seen when these activities were measured in broth-grown CV and CAC.

Virulent *L.pneumophila*, therefore, not only elicit a reduced

oxidative burst, they are more resistant to the toxic products than avirulent legionellae. The studies reported by Jepras & Fitzgeorge (1985) also showed that two avirulent strains of *L.pneumophila* differed in their resistance to killing by singlet oxygen, a toxic product of the xanthine oxidase system. Such differences could explain the killing of CAC by guinea pig alveolar macrophages compared to the intracellular survival of CA in the present study.

The levels of catalase and superoxide dismutase produced by intracellular CV are not known but it is possible that production may be enhanced on exposure to the toxic oxygen species. *Salmonella typhimurium* responds to the presence of hydrogen peroxide by synthesising stress-induced proteins which aid the intracellular survival of the bacterium. A similar stress-induced system may operate in *L.pneumophila* producing proteins such as catalase which protect against the oxidative attack. It is apparent from these studies and others (Abu-Kwaik *et al.*, 1993) that *L.pneumophila* secretes a different combination of proteins following incubation with macrophages to that produced by growth on culture medium. Identification of proteins produced only on exposure to the intracellular environment could reveal more possible virulence factors. Further information may also be obtained by examining the oxidative response of alveolar macrophages to virulent/avirulent *L.pneumophila* in the early stages of experimental infection. The simultaneous measurement of phagocytosis and phagocytosis-induced respiratory burst is possible using fluorescent markers and flow cytometry. Bass *et al* (1983) described a technique which quantifies the respiratory burst of individual PMNLs as the oxidation (by hydrogen peroxide) of 2'7'dichlorofluorescein diacetate (DCF-DA) to the highly fluorescent 2'7'dichlorofluorescein (DCF). Szejda *et al.*, (1984) used this technique and Texas Red-labelled bacteria to correlate phagocytosis

with the resulting respiratory burst of PMNLs. Using this dual fluorescence technique it may be possible to investigate the oxidative response of macrophages and PMNLs taken directly from infected guinea pigs following aerosol challenge with CV, CA and CAC.

Inhibition of phagosome-lysosome fusion has been reported as the critical property in allowing intracellular replication of a strain of *L.pneumophila* compared with its' avirulent counterpart. (Horwitz, 1987) but this may not always be the case since virulent legionellae are known to allow fusion and still multiply (this study, Rechnitzer & Blom, 1989). Thus, while prevention of contact with lysosomal enzymes may be a virulence mechanism for some legionellae it may be resistance to lysosomal contents which is important in others. Experiments which measure the levels and toxicity of lysosomal enzymes released following incubation with strains of differing virulence are required.

Survival of *L.pneumophila* within the hostile environment of macrophages is important but it has been shown here and elsewhere that survival is not sufficient. Avirulent strains were able to withstand 48 hours inside macrophages in the *in vitro* assay yet their inability to multiply meant that the defences of the guinea pig were sufficient and no disease resulted. The focus of research should perhaps move towards finding out how virulent strains are able to grow intracellularly rather than how they resist killing. It has been suggested that the availability of iron from the labile iron pool of monocytes is a critical determinant for intracellular replication by *L.pneumophila* (Byrd and Horwitz, 1989). Avirulent strains have been shown to have a greater (4-fold) requirement for iron than their virulent counterparts for growth *in vitro* (Johnson *et al.*, 1991) despite the fact that both use similar ferric reductases to obtain the iron (Poch & Johnson, 1993). The ferric reductase of avirulent legionellae showed a 2-fold increased activity when NADPH was used as reductant (compared

with NADH) and the authors speculated that this may affect utilisation of iron intracellularly due to the decrease in NADPH during phagocytosis. There is no evidence to suggest that the legionellae require NADPH from the intracellular environment of the macrophages and, since the enzymes were identical in every other respect, the extra requirement for iron by avirulent strains has not been explained. The quantity of available iron might therefore distinguish the ability of virulent and avirulent strains to multiply intracellularly, since the virulent forms are able to utilise smaller quantities. Thus, regardless of the ability to survive intracellularly, the virulent strains have the advantage through being able to utilise intracellular iron.

In summary, there are a number of differences between virulent and avirulent strains which are related to their ability to cause LD experimentally. Virulent forms reduce the anti-microbial reactions of phagocytic cells, are better able to resist the products of the oxidative response and, utilise intracellular iron more efficiently than their avirulent counterparts.

Events occurring in the early stages of infection with *L.pneumophila* are thus becoming clearer and with improvements in the genetic manipulation of the organisms and realisation of the need to study bacteria growing intracellularly the principal factors which control these events should soon be identified. Inhibition of such factors would certainly prevent disease and, if a vaccine were warranted for a disease which has a lower global mortality than many diseases for which there is no vaccine, one could be developed with this knowledge.

6.2 PATHOLOGY OF LD

The processes occurring following multiplication of *L.pneumophila* in the lungs are less well-defined than those discussed in section 6.1., although

many have been proposed. Given the difficulties in prompt diagnosis of LD they are equally important, serving to define goals for effective therapy. The evidence for and against the role of TDP in the disease was discussed in Chapter 4 but, considering the possible importance of the enzyme, merits further discussion here. There is a large discrepancy between the findings of work performed on TDP at this laboratory and that of Blander *et al.*, 1990. The results of work performed at CAMR have shown that the enzyme is responsible for the damage to lung tissue which is seen during LD but the American workers provide evidence which suggests that TDP is not required for deaths, typical pathology and progression of disease to occur. This was concluded by the fact that a mutant strain of *L.pneumophila* which did not produce the protease was as virulent for guinea pigs as the parent strain from which it was derived. When these strains were examined in the guinea pig aerosol infection model in the present studies differences in the interpretation of results were apparent, the most striking of which was the fact that both parent and mutant had been reported to be highly virulent for guinea pigs but very high doses of the strains were required to cause disease. At these high doses both strains caused disease and death in guinea pigs but the virulence and progression of disease was much less severe than strains normally used to cause LD in this model. There was also a slight difference in the virulence of the strains in that the LD₅₀ and average survival time of the mutant were greater than the parent. The results of histopathological analysis of lung sections following infection in the two models showed an important discrepancy. In the experiments of Blander *et al* (1990) the authors concluded that the enzyme could not be responsible for the characteristic pathological lesions of LD because the mutant strain caused identical lesions to the parent strain. When compared in the CAMR model of LD the lesions caused by the mutant strain were much less severe than those caused by the parent.

Such reduced necrosis caused by infection with the mutant strain supports the role of the enzyme in contributing to the pathogenesis of disease. The mutant is known to produce very small quantities of proteolytically active TDP which may be responsible for some of the pathology and there was no mention of this in the Blander *et al* publication. The discrepancies between the evidence obtained using this mutant in the two laboratories sheds doubt on the role of TDP *in vivo* but it is important to consider that the evidence based on this mutant stands alone in supporting a theory that the enzyme does not have a significant role in the pathogenesis of LD. There is, on the other hand much evidence, both direct and circumstantial which supports a role for TDP. The cytotoxicity of the enzyme has been characterised genetically and purified TDP is known to cause lesions identical to those seen during LD. Quantities of proteolytically active enzyme sufficient to cause such lesions have been detected *in vivo* in guinea pigs and in human macrophages. *Legionella micdadei* does not produce the enzyme and causes disease in humans less frequently than *L.pneumophila* and infection only results in a severe form of illness in immunocompromised individuals. The properties of the enzyme are such that it degrades substances which may directly or indirectly contribute to limiting the spread of *L.pneumophila* in the lungs and an inhibitor which neutralised the activity of TDP caused an increase in survival when given to guinea pigs but did not prevent death. Perhaps the use of animal models which use death as an end point are too severe a test of the role of the enzyme.

There is little doubt that TDP can cause the lung damage which is seen during LD but this does not exclude other toxic substances produced by *L.pneumophila* eliciting similar damage in the absence of TDP. The TDP-minus mutant examined in the present studies caused a pathology typical of LD in guinea pigs, but it is probable that, in this instance, other

factors contributed to the lung damage. These may have been bacterial or host derived.

There are other substances produced by legionellae in artificial culture which have disease-causing properties but the role of these during experimental or human infection has yet to be established. One such substance is a low molecular weight (3400 kDa), toxin which was first described by Friedman (1980) and Hedlund (1981) and was shown to inhibit human neutrophil function (Hedlund, 1981, Friedman *et al.*, 1982 Lochner *et al.*, 1985). The toxin has received little attention since and there is no evidence that the toxin is produced by *L.pneumophila* growing *in vivo* at the concentrations required to exert the inhibitory effects on neutrophils. In the initial reports of the toxin the purified form was shown to have a lethal toxicity for mice and on isolated mouse macrophages (Hedlund, 1981) but the mechanism of this toxicity was not reported. An enzyme which has haemolytic activity distinct from that of TDP has been described (legiolysin, Rdest *et al.*, 1991). It has no proteolytic activity but could have the same haemolytic activity as TDP and contribute to the lung damage. Enzymes produced by PMNLs are known to cause pathological effects in the lungs (Janoff, 1985) but it is thought that these are not an important cause of lung damage seen in LD because guinea pigs with induced neutropenia exhibited typical LD pathology following aerosol challenge with virulent *L.pneumophila*. Although not a major cause, PMNL enzymes may still be able to contribute to the lung damage under normal circumstances. A combined attack by host and bacterial toxins and enzymes is therefore feasible and may explain why a similar pathology can occur when one component e.g. TDP is missing. Aside from the tissue destructive action, there are also several enzyme activities demonstrated for *L.pneumophila* which could have the same overall properties of TDP in the absence of the latter. An example is the acid phosphatase produced by

L.micdadei which inhibits superoxide anion production (Saha *et al.*, 1985). *L.pneumophila* also produces an acid phosphatase (Conlan *et al.*, 1986) which may have the same properties as that secreted by *L.micdadei*. The different activities of TDP which have been proposed as pathogenic mechanisms of *L.pneumophila* could therefore possibly be accounted for by other enzymes/toxins of bacterial or host origin. However, as TDP has all these properties and is produced *in vivo* in sufficient quantities to exert the proposed effects, it is a strong candidate for the principal disease mediator and it is difficult to ignore the effects which neutralisation of the enzyme may have on the prognosis of patients with LD.

Enzymes with activities/properties different to those of TDP are also being discovered. *L. micdadei* produces protein kinases which may have a role in modulating anti-microbial processes by phosphorylation of host proteins (Saha *et al.*, 1988) although no relevant protein substrate has been identified and similar activity has not been found in *L.pneumophila*. An ADP-ribosyltransferase has been detected and purified from lysates of *L.pneumophila* and although its precise function has not been identified it is thought to have a similar activity to exotoxin S, an important virulence factor of *Ps.aeruginosa* (Belyi *et al.*, 1991).

Thus, numerous mechanisms by which *L.pneumophila* prevents the host from eradicating the infection have been proposed. There is little doubt that more than one factor is responsible for the pathogenesis of the disease and understanding the situation as a whole is complicated by the large number of strains of *L.pneumophila* which have been studied. This was portrayed in Chapter 3 by the difference in virulence between the Corby strain used in these studies and the Philadelphia 1 strain which has been the basis of much experimentation. It is difficult to compare the findings of experiments on bacteria which exhibit vast differences in virulence.

6.3 ANIMAL MODELS OF LD.

The use of experimental models of infection is also variable and much of the information regarding virulence has been performed either in tissue culture of cells which were not alveolar in origin or in animal models using routes of administration which were inappropriate to the airborne nature of contraction. The susceptibility of SCID mice which had been given a human lymphocyte system to infection with *L.pneumophila* could result in more definitive investigations where the interaction of the bacteria with the cells it encounters in human disease can be studied in a whole animal model.

6.4 ENVIRONMENTAL FACTORS-ROLE OF AMOEBAE

L.pneumophila is a self limiting, opportunistic pathogen, man is its only natural animal host and there are no means for its persistence in man. Person to person transmission does not occur and the patient either dies or eradicates the bacteria. The bacteria can survive very successfully in low-nutrient water environments and the strategies it must employ to multiply and be competitive in such an environment, may also be those which allow it to survive and multiply in the harsh circumstances of the alveoli. Comparisons between replication of legionellae within amoebae in water and macrophages in alveoli have often been made and it is tempting to suppose that mechanisms which allow survival of the killing mechanisms of amoebae are the same as those which permit survival and replication within macrophages. There is some evidence to support this assumption but the processes occurring following uptake of legionellae by amoebae are only just beginning to be studied at a molecular level. One report has suggested that the amoeba *Acanthamoeba polyphaga* exhibits an oxidative response comparable to that of neutrophils when virulent and avirulent legionellae are phagocytosed by either cell (Halablab *et al.*, 1990). Since

mutants defective in the mip protein, are deficient in intracellular replication in amoebae in a similar manner to that in macrophages (Cianciotto & Fields, 1992), it is possible that similar events occur in the early stages of infection of amoebae as in macrophages and that *L.pneumophila* employs the same strategy to overcome them in both. It was mentioned in Chapter 3 that the proteins expressed by *L.pneumophila* change following intracellular replication within amoebae and that this also occurs following infection of macrophages. Since amoebae ingest bacteria to digest as a food source, resistance of legionellae to the digestive enzymes would be required and this may be related to resistance to lysosomal enzymes in macrophages. However Fields (1993) has proposed that *L.pneumophila* avoids exposure to lysosomes by multiplying within the smooth or rough endoplasmic reticulum of the cell, analogous to the phagosome-lysosome fusion inhibition theory. Further studies are clearly required and care must be taken to avoid generalisations about a bacterium which has a complex pathogenesis. It should be remembered that other species of *Legionella*, which rarely cause human disease are able to multiply within amoebae suggesting that intracellular replication within amoebae is not an indicator of virulence in humans. The route from water to the respiratory tract is prone to a range of influences and, in order to cause disease, a combination of factors are important not only in the environment and in the lungs but also during transmission from one to the other.

6.5. CONCLUSION.

To conclude, the studies presented in this thesis have confirmed previous work, supported current work by others and added new information regarding the nature of *L.pneumophila* growing *in vivo* and the

response of a guinea pig host to virulent and avirulent bacteria. Further evidence supporting the role of the tissue destructive protease has been provided and a novel animal model incorporating human cells described. The virulence and pathogenic mechanisms of *L.pneumophila* have been discussed and it is apparent that several bacterial, host and environmental factors are involved. Avirulent forms of *L.pneumophila* produced by multiple passage on artificial media have limited use as tools for elucidating virulence factors of the organism since the changes which result in the loss of virulence are multiple and appear to vary depending on the strain of *L.pneumophila* used. Genetic manipulation of *L.pneumophila* has provided information regarding certain proteins which are a part of the disease process but genetic techniques will only be of use if performed on strains which are virulent in relevant models of the disease.

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